Bead-based quantification of mucosal lymphocytes

Absolute quantification of cell subsets in selected mucosal samples was performed using a fluorescent bead-based assay modified from a peripheral blood assay optimized in our laboratory.[2-4] Briefly, biopsy specimens were weighed and then, after processing, all isolated cells were resuspended in PBS supplemented with 2% FCS (Sigma-Aldrich, St. Louis, MO). Each cellular suspension was then spiked with 10 µm SPHERO AccuCount Rainbow Fluorescent Particles (Spherotech, Lake Forest, IL) at a concentration of 1,000 beads/mg of absolute tissue weight (measured before processing). Polychromatic flow cytometry staining was then performed on each sample with LIVE/DEAD Aqua dye and combinations of antibodies to CD3 (APC-Cy7 conjugate), CD4 (PE conjugate), CD8 (Alexa 700 conjugate), CD45 (FITC conjugate), HLA-DR (PE-Texas Red conjugate), NKG2A (Pacific Blue conjugate), and/or NKp44 (PE conjugate).

Absolute counts of each cell type were determined using the following formula:

total cells / mg of tissue = (# of events in gated cell region / # of events in gated bead region) x (total beads added to sample / mass of sample (mg))

Assays were performed using duplicate biopsy preparations and the results were then averaged. The full methodology and optimization of this assay is described elsewhere.[4]

Intracellular cytokine staining assays

To assess mucosal NK cell subset function, we performed *ex vivo* functional analyses optimized in our laboratory using antibodies titered and tested to be cross-reactive in macaques. Briefly, 1 x 10^6 mononuclear cells were resuspended in RPMI

1640 (Sigma-Aldrich) containing 10% FBS (R10) and either stimulated with PMA (50 ng/ml) and ionomycin (1 µg/ml) or cultured in medium alone. Alternatively, cells were stimulated with the MHC-devoid cell line, 721.221, as described previously.[3] Anti-CD107a (PerCp-Cy5 conjugate, clone H4A3) was added directly to each of the tubes at a concentration of 20 µl/ml and Golgiplug (brefeldin A) and Golgistop (monensin) were added at final concentrations of 6 µg/ml, then all samples were cultured for 12 h at 37°C in 5% CO_2. After culture, samples were surface-stained using markers to delineate live cells (LIVE/DEAD Aqua dye, Invitrogen), leukocytes (CD45), and NK cell subpopulations (CD3, NKG2A, NKp44). Cells were then permeabilized using Caltag Fix & Perm and intracellular cytokine staining was performed for combinations of MIP-1β (FITC conjugate, clone 24006, R&D Systems), IFN-γ (APC conjugate, clone B27, Invitrogen), TNF-α (Alexa 700 conjugate, clone Mab11) and IL-17a (Alexa 647 conjugate, clone eBio64DEC17, eBioscience, San Diego, CA). Production of IL-17 and IFN-γ by mucosal $CD4^+CD3^+$ T cells was evaluated using the same conditions. All reagents were purchased from BD Biosciences unless otherwise stated.

RNA isolation from sorted cells and quantitative RT-PCR

$NKG2A^+$ and $NKp44^+$ cells were sorted into 10% RPMI. Half of the cells were stimulated with calcium ionomycin and PMA (50 ng/ml) for 2 hrs at 37°C. Stimulated and unstimulated cells were washed once in ice cold PBS, and RNA extracted and cDNA prepared using the TaqMan PreAmp Cells-to-CT kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's directions. Preamplification of cDNA was carried out using a pool of TaqMan gene expression assays (Applied Biosystems)

for the targets of interest diluted to 0.2x concentration. cDNA samples were preamplified for 18 cycles following the manufacturer's protocol, and preamplified cDNA was diluted 5-fold with 1xTE and then subjected to real-time PCR. Each real-time PCR reaction contained 10 µl of 2x TaqMan Gene Expression Master Mix, 1 µl of 20x Taqman Gene Expression Assay, 4 µl of water, and 5 µl of diluted Preamp cDNA. All real-time PCR assays (supplemental Table 2) were designed to amplify rhesus macaque sequences and were purchased from Applied Biosystems. Quantification of RORC was performed using the forward primer—5'-TGAGAAGGACAGGGAGCCAA-3'; reverse primer—5'-CCACAGATTTTGCAAGGGATCA; and MGB probe—6FAM-5'-TCATGAGAACACAAATTGA-3'-TAMRA. Thermal cycling was carried out using an Applied Biosystems 7900HT Real-Time PCR System, with a UDG hold for 2 min at 50°C, enzyme activation for 10 min at 95°C, and then 40 amplification cycles at 95°C for 15 sec and 60°C for 1 min. The linearity of all PCR assays was verified over a 3-4 log range of input cDNA. Controls included no template controls and no RT controls. Input cDNA was normalized based on the average expression of 2 endogenous control genes (importin 8 and polymerase RNA II polypeptide A); these genes were selected from a panel of 4 endogenous controls using the geNorm algorithm [1] to analyze the stability of endogenous control genes in sorted NK cells. Relative gene expression (RGE) was calculated using the formula

$RGE=1000 \times 2^{[-(CT_{(Target)}-CT_{(mean\ Endogenous\ Control)})]}$.

Quantification of cell-associated SIV RNA

Quantification of cell-associated SIV RNA was performed using the forward primer: U5PBSF—5'-GAAACCGAAGCAGGAAAATC-3'; reverse primer: U5PBSR—5'-

CTGCCTTCACTCAGCCGTACT; and probe: U5PROBE—6FAM 5'-AGGAGTCTCTGACTCTCCTTCAAGTCCCTGTT-3' TAMRA (Applied Biosystems). This amplicon is designed to amplify a 92 bp fragment of full length SIV RNA that spans the U5 region of the 5'LTR and the primer-binding site.

To determine the SIV copy number in sorted cells, the SIV primers and probe were included in the preamp reaction. Results were normalized using *in vitro* transcripts of the SIVmac239 PBS-U5 region; the sensitivity of the assay was 10 copies. Two-fold serial dilutions of SIV RNA, starting at 1000 copies, were spiked into 1 ng cDNA from PBMC of uninfected animals and preamplified for 18 cycles. The preamplified samples were diluted 5-fold and used to generate a standard curve to determine the SIV RNA copy number in sorted cells.

Table S1. Antibodies used for phenotypic analyses

Antibody	Clone	Fluorochrome	Manufacturer
anti-caspase-3	C92-605	Alexa647	BD Biosciences (La Jolla, CA)
anti-CCR5	3A9	PerCp-Cy5.5	BD Biosciences
anti-CCR6	11A9	PE	BD Biosciences
anti-CCR7	150503	Alexa700*	R&D Systems (Minneapolis, MN)
anti-CD3	SP34.2	APC, APC-Cy7	BD Biosciences
anti-CD4	L200	PE	BD Biosciences
anti-CD8α	RPA-T8	Alexa700	BD Biosciences
anti-CD16	3G8	Alexa700, PE, FITC	BD Biosciences
anti-CD20	L27	PerCp-Cy5.5	BD Biosciences
anti-CD45	D058-1283	FITC	BD Biosciences
anti-CD45	Tu116	PerCp-Cy5.5	BD Biosciences
anti-CD45	MB4-6D6	APC	Miltenyi Biotec (Gladbach, Germany)
anti-CD56	NCAM16.2	PE-Cy7	BD Biosciences
anti-CD69	TP1.55.3	PE-Texas Red	Beckman-Coulter (Fullerton, CA)
anti-CD117	104D2	PE-Cy7	BD Biosciences
anti-CD127	R34.34	PE	Beckman-Coulter
anti-CXCR3	1C6/CXCR3	PE-Cy5	BD Biosciences
anti-HLA-DR	Immu-357	PE-Texas Red	Beckman-Coulter
anti-Ki67	B56	FITC	BD Biosciences
anti-NKG2A	Z199	Pacific Blue*	Beckman-Coulter
anti-NKp44	Z231	PE, PerCp-Cy5.5	Beckman-Coulter
anti-NKp44	2.29	APC	Miltenyi Biotec
anti-NKp46	BAB281	PE	Beckman-Coulter
anti-perforin	Pf-344	FITC	MabTech (Mariemont, OH)
annexin-V	N/A	FITC	Biovision (Mountain View, CA)

* In-house custom conjugate

Table S2. Real-time PCR assays used for transcriptional analysis

Gene symbol	Assay ID
AHR	Rh02839281_m1
CCL20	Rh02788116_m1
CCL3	Rh02788104_gH
CCL5	Rh02788105_m1
IDO1	Rh02787165_m1
IFN-A1	Rh03456707_gH
IL-1β	Rh02621711_m1
IL-12A	Rh02621733_m1
IL-17A	Rh02621750_m1
IL-22	Rh02877345_m1
IL23A	Rh02872166_m1
IL-6	Rh02789322_m1
IPO8	Hs00183533_m1
Nfil3	Rh02915951_S1
PGK1	Hs99999906_m1
PolR2A	Hs00172187_m1
RORA	Rh00931148_m1
RORC*	Custom
TBP	Hs99999910_m1
TGFB1	Rh02621726_m1

* Unless otherwise noted, all assays were purchased from Applied Biosystems.

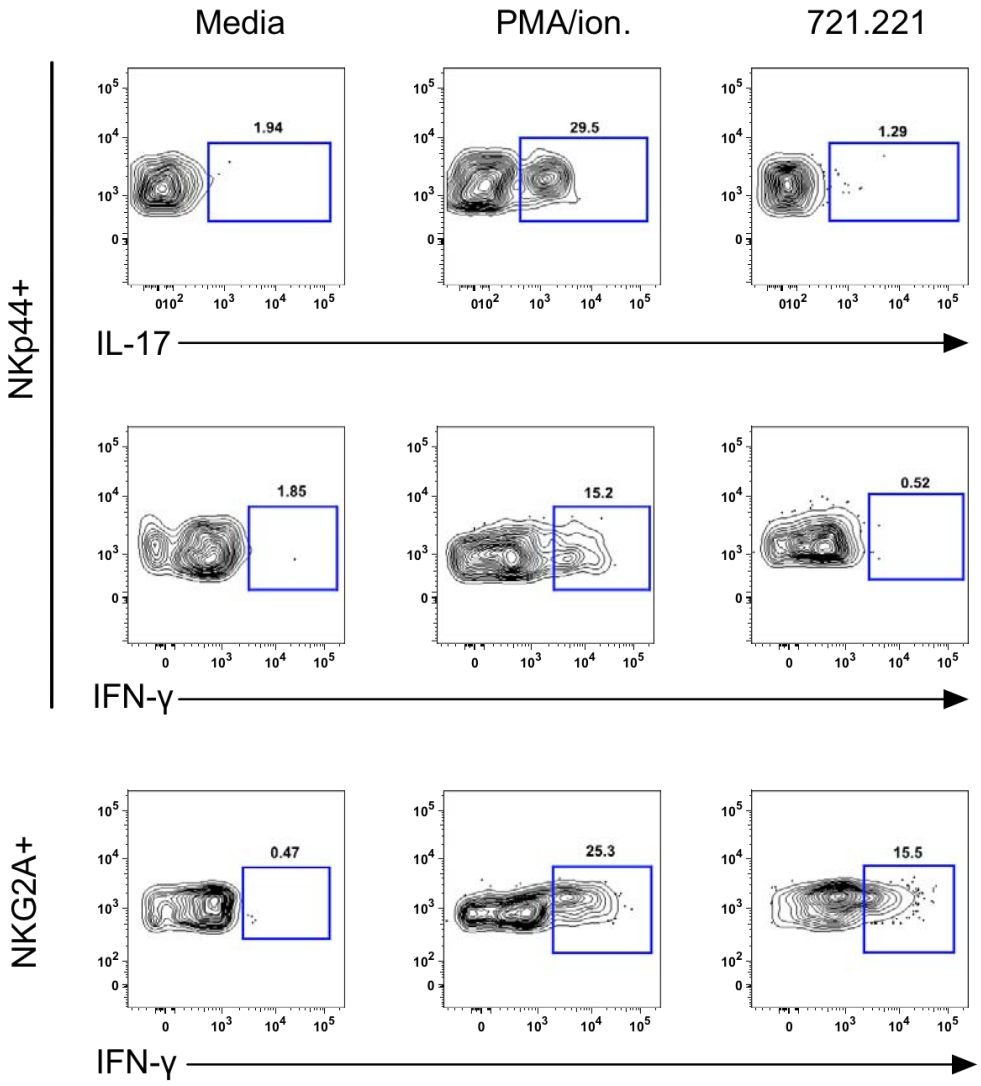

Figure S1. NKG2A^{+}, but not NKp44^{+} NK cells, respond to MHC-devoid 721.221 cells. Mononuclear cells isolated from colorectal biopsies of normal rhesus macaques were stimulated with either PMA/ionomycin or 721.221 cells at a 2.5:1 effector-to-target cell ratio for 12 hours, and then analyzed for intracellular production of IL-17 or IFN-γ.

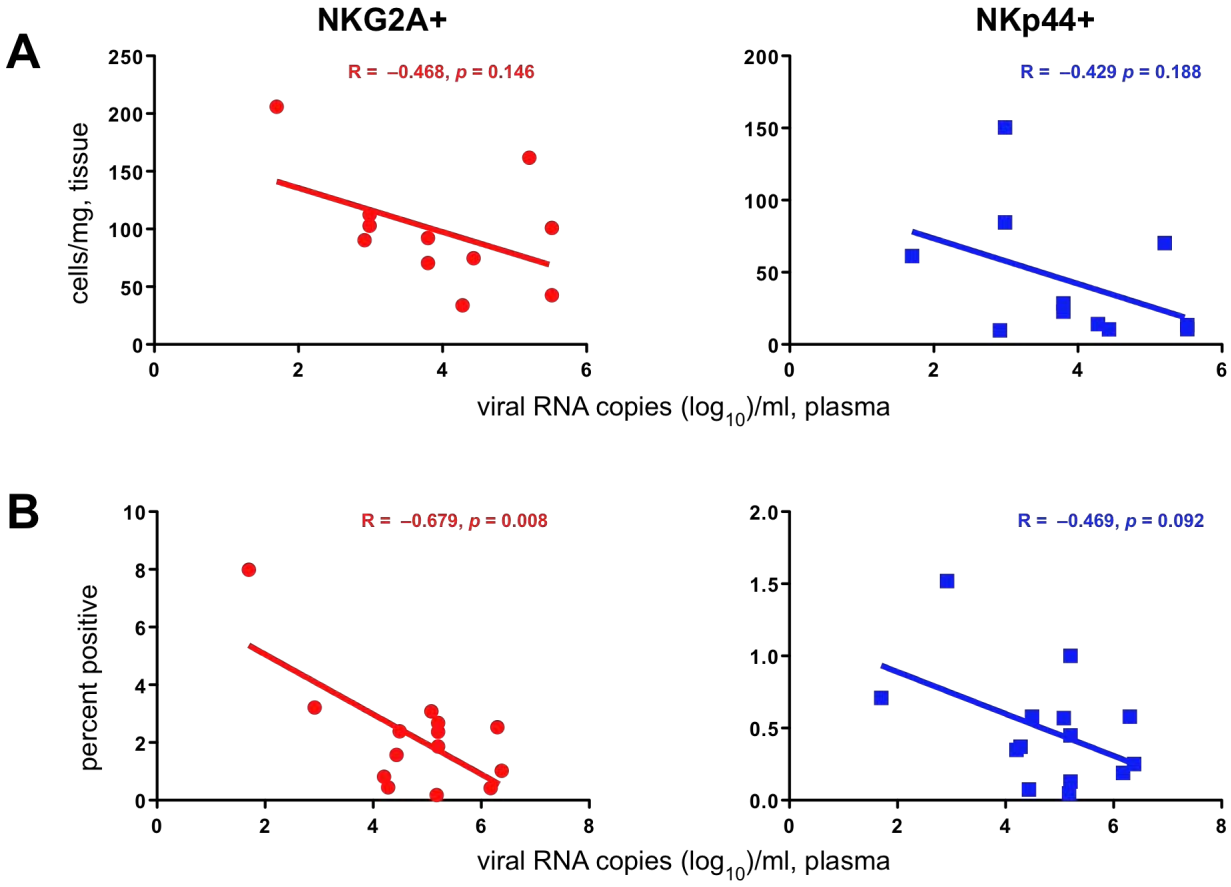

Figure S2. Relationships of NKp44$^+$ and NKG2A$^+$ NK cells in the gut mucosa to plasma viral loads. Absolute numbers **(A)** and frequencies **(B)** of NKp44$^+$ and NKG2A$^+$ NK were correlated with plasma viral loads. *P* values < 0.05 are considered significant.

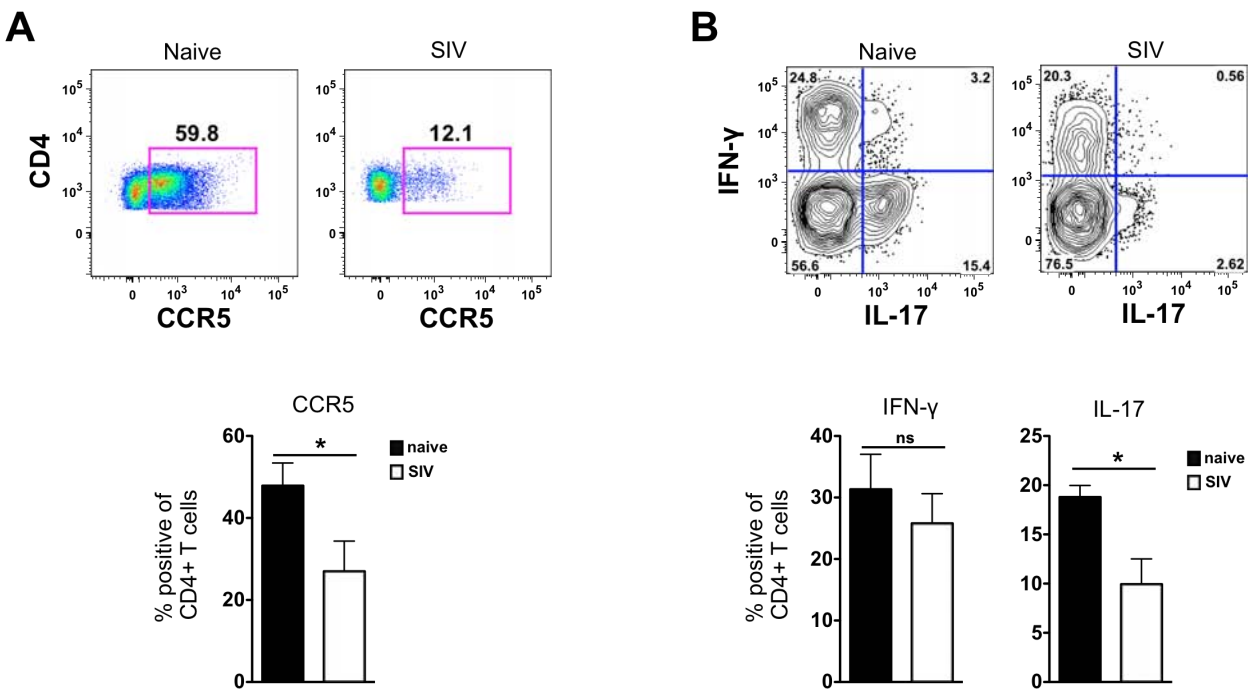

Figure S3. Chronic SIV infection depletes CCR5$^+$ CD4$^+$ T cells and CD4$^+$ Th17 cells in the gut. (A) Frequencies of CCR5$^+$ CD4$^+$ T cells were determined among live CD45$^+$ mononuclear cells isolated from colorectal biopsies. **(B)** Frequencies of IFN-γ- and IL-17-producing CD4$^+$ T cells in naïve and SIV-infected macaques were determined using a 12-hour ICS assay as described in the Methods. Bars represent means ± SEM of 6 to 8 animals per group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Mann-Whitney *U* test.

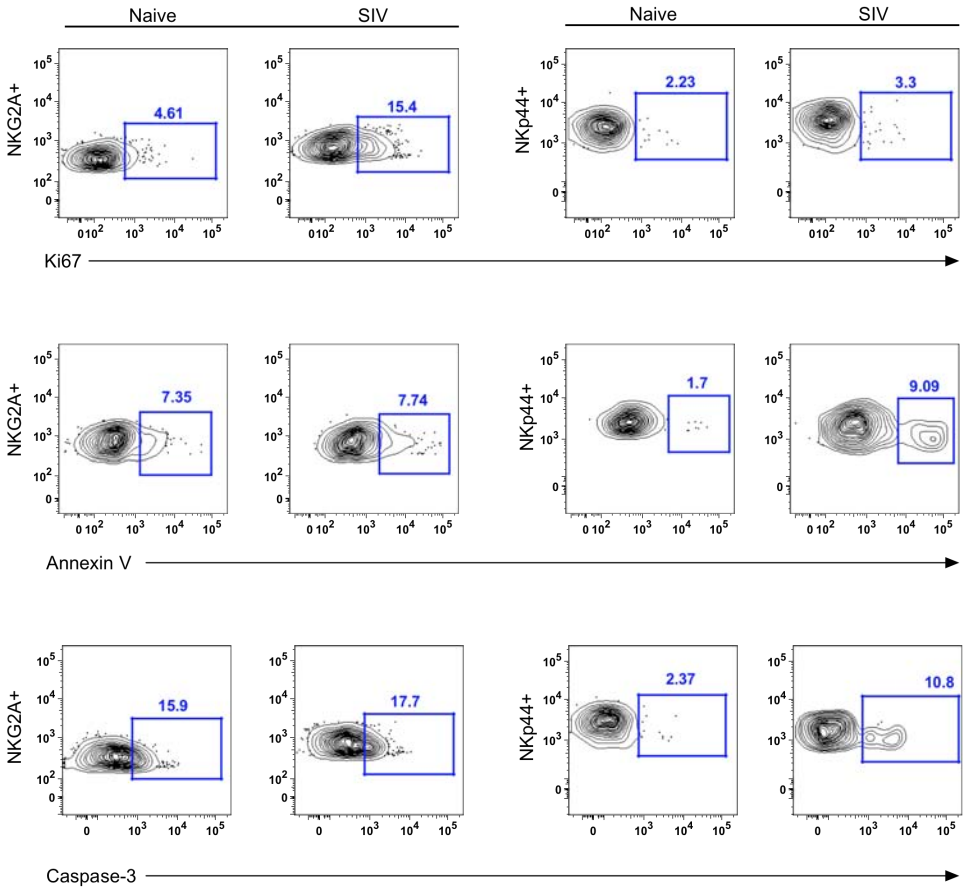

Figure S4. Chronic SIV infection causes an upregulation of Ki67 and apoptotic markers in NKG2A$^+$ and NKp44$^+$ NK cells. Representative flow cytometry plots of intracellular Ki67 and caspase-3 or surface expression of Annexin V-binding on NKp44$^+$ and NKG2A$^+$ NK cells from naïve and SIV-infected macaques.

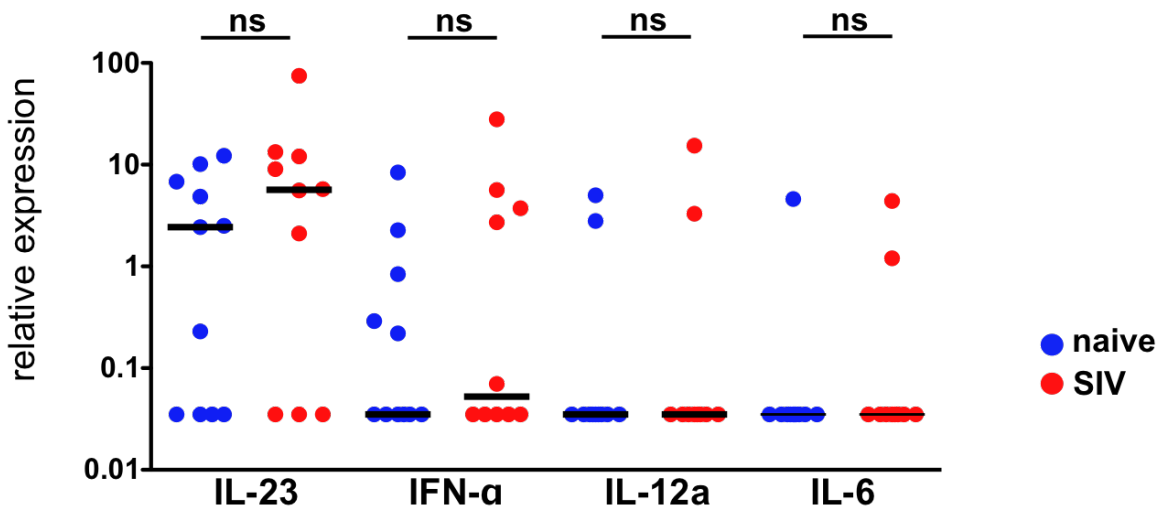

Figure S5. Cytokine expression in biopsy specimens from naïve and SIV-infected macaques. Quantitative RT-PCR analysis of IL-23, IFN-α, IL-12a, and IL-6 transcripts was performed on rectal biopsies collected from naïve and SIV-infected macaques and processed as described in the Methods. Lines indicate medians of 10 to 12 animals per group. Mann-Whitney *U* tests were used for naïve-versus-SIV comparisons. *P* values < 0.05 are considered significant.

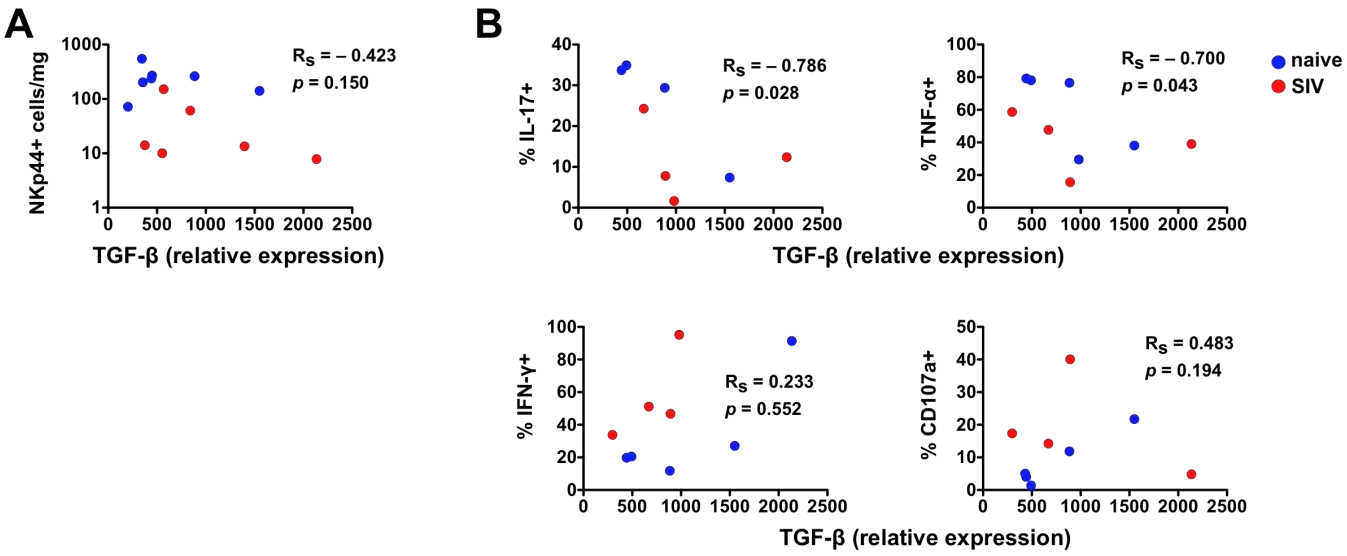

Figure S6. TGF-β is upregulated in the gut mucosa of SIV-infected macaques and is associated with a loss of function in NKp44$^+$ NK cells. Relative expression of TGF-β in whole biopsy pieces was correlated with **(A)** absolute numbers of NKp44$^+$ NK cells and **(B)** NKp44$^+$ NK cell effector functions in biopsy specimens of the same animals. *P* values < 0.05 are considered significant.

References

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4. Reeves RK, Evans TI, Gillis J, et al. Quantification of mucosal mononuclear cells in tissues with a fluorescent bead-based polychromatic flow cytometry assay. J Immunol Methods. 2011;In press.