Online Material and Methods

Colorectal tissues processing for flow cytometric analysis

Briefly, the colonic tissue pieces/rectal pinches were incubated with Hank's balanced salt solution containing 5 mM EDTA and 2 mM DTT at room temperature for 15 minutes twice. The supernatants, which contain intraepithelial lymphocyte cells, were collected, pooled and filtered through 100 mM cell strainers. The collected cells were called intraepithelial lymphocytes (IEL) in the text. The tissues were then incubated with Liberase (Roche) at 37°C for 30 min. After enzyme digestion, the tissue chunks were mashed through a syringe end and filtered through a 100 µm cell strainer. The collected cells were called lamina propria (LP) in the text.

Tissue processing for Env specific memory B cells, PB, PC flow cytometry assay

Rectal biopsies were rinsed with pre-warmed RPMI1640 (Invitrogen) containing 2×antibiotic–antimycotic solution, 2-mM L-glutamine (Invitrogen) and 2 ug/ml Collagenase (Sigma–Aldrich). Prior to incubation (25 min at 37°C) the pinches were minced using a scalpel and a 19G needle, transferred in 10 ml of the same media to a 50 ml tube and pulse vortexed every 5 min. The digested tissue was passed 5 times through a blunt end cannula. The liberated cells and tissue debris were passed through a 70 µm cell strainer, and the cells were washed in R10 (RPMI1640 containing 2×antibiotic–antimycotic solution, L-glutamine and 10% FBS) prior to staining. Cells (1-2x10^6/tube) were used to stain with the antibody mixture. After a 25min surface staining and envelope protein staining, cells were washed with 2% FBS in PBS, fixed and permeabilized for 15 min at room temperature using a transcription buffer set for IRF-4 (BD Bioscience, San Jose, CA). After washing in Permwash solution, intracellular staining was conducted. Subsequently, cells were washed and resuspended in PBS and acquired within 2 hours on a custom 4-laser LSR II (BD Bioscience). Samples were diluted in 1xPBS and were passed through a 35 µm cell strainer. A minimum of 50000 live cells in the lymphocytic gate were acquired in DIVA. The flow cytometry staining panels were listed in supplementary Table7.

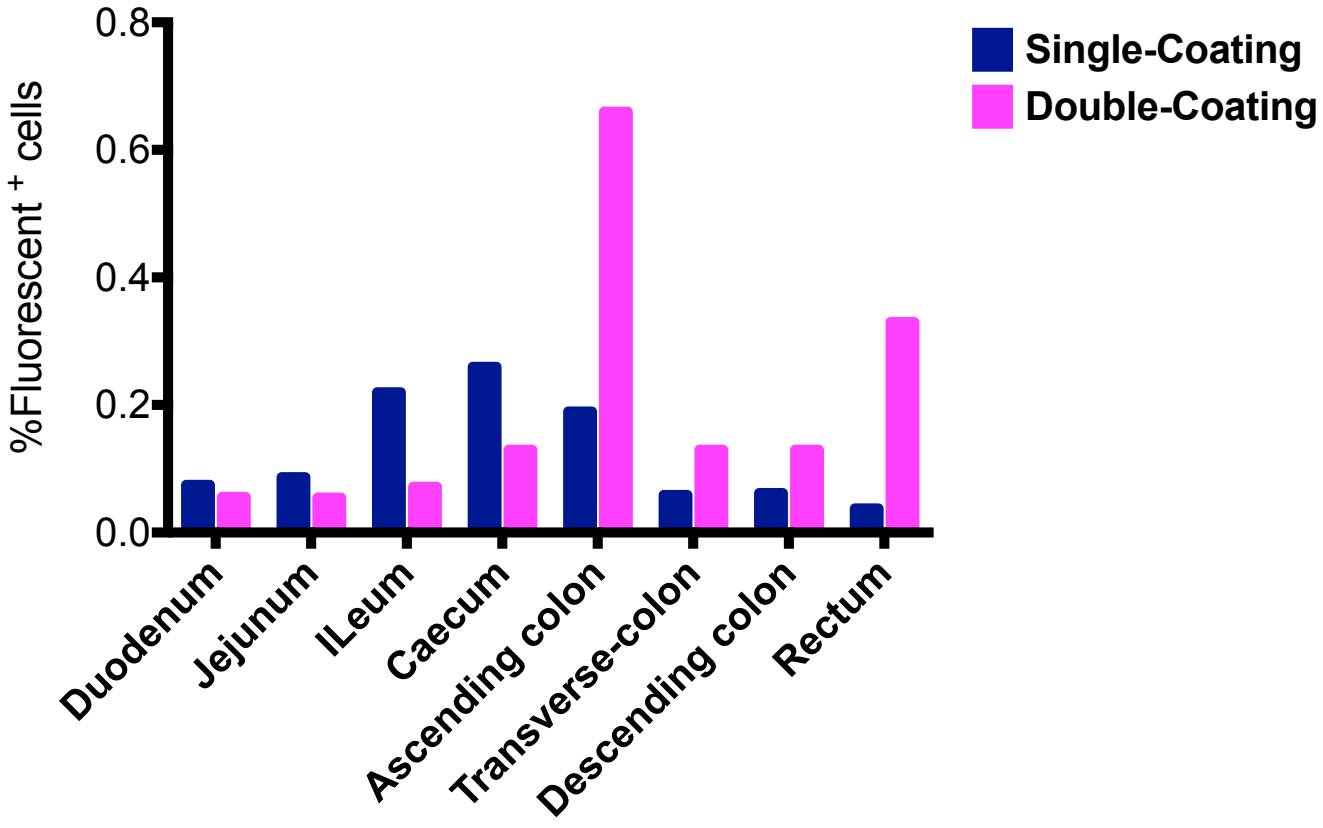
Sources of antibodies

The following antibodies were purchased from BD Pharmingen: CD3-PE-Cy7, CD8-APC-Cy7, Ki67-APC, HLA-DR-PE-Cy5, CCR5-PE, CD45-Percp-cy5.5, HLA-DR-APC-Cy7, Lin-FITC, CD11b-PE-Cy5, CD21-PE-Cy7, IgG-APC-Cy7, Ki67-Alexa700; from Biolegend: CD28-FITC, CD95-PE-Cy5, IFNγ-Alexa Fluor® 700, IL-2-Alexa Fluor® 647, TNFα-PE, CD69-Alexa Fluor® 700, CD15- Alexa Fluor® 700, CD14-BV711, CD8-BV785, CD16-V450/BV421, CD138-PE; from StemCell: CD38-FITC; from eBiosciences/Invitrogen: CD4-Qdot605, CD2-Qdot605,CD14-Qdot605, HLA-DR-Qdot800; From Beckman Coulter: CD19-PE-Cy5, From Southern Biotech: IgD-Texas Red, and from Miltenyi Biotech: CD33-PE.

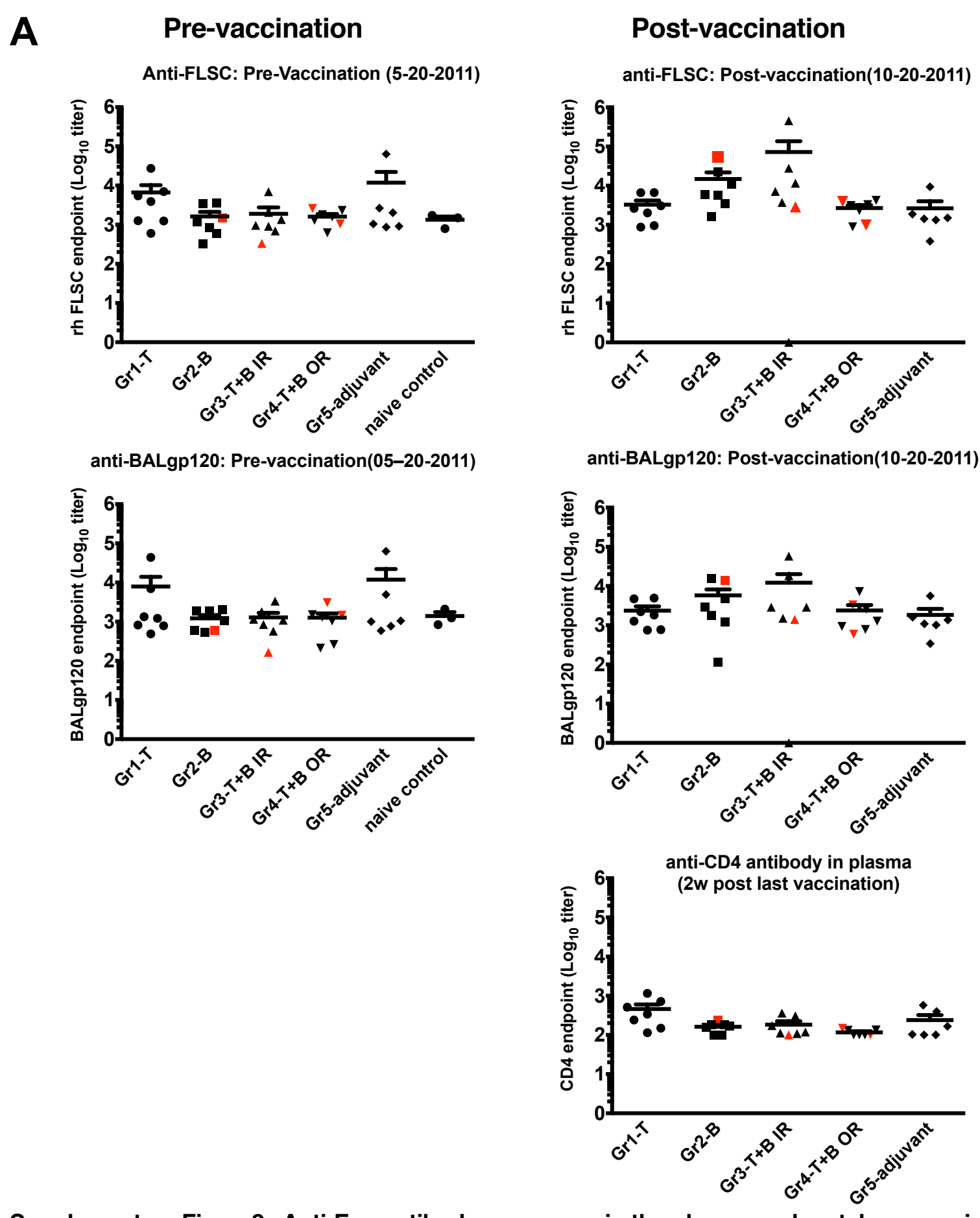
Sample processing for rectal secretions and rectal tissue explants to measurements rhFLSC binding antibodies

Rectal biopsies (3 mm) were obtained using a radial Jaw disposable biopsy instrument inserted into the rectum 3–5 cm. Biopsies were obtained circumferentially and placed in RPMI1640 medium. Biopsies were washed and cultured in sodium bicarbonate buffered RPMI (5 ml 7.5% Na2HCO3 (Invitrogen)/500 ml R10) at 37 °C and 5% CO2 in 48-well plates. Four pinches per well were cultured. Medium was replaced with 0.6 ml fresh R10 at the end of the day. Supernatants were collected the following day (day 1) centrifuged and preserved at −20 °C for further analysis. Rectal secretions were collected using cotton-tipped swabs and then stored at −70 °C in 1 ml of 1 × PBS buffer containing 0.1% BSA, 0.01% thimerosal, and 750 Kallikrein inhibitor units of aprotinin. Rectal swabs were thawed and the recovered solution was passed through a 5 µm PVDF microcentrifugal filter unit (Millipore, Billerica, MA) in order to remove contaminating particles. The buffer flow-through was collected and stored at −20 °C until analysis.

Measurements of rhFLSC binding antibodies in rectal secretions and rectal tissue explants
Specifically, 96-well ½ area plates (Greiner) were incubated overnight at 4°C with α-Mon IgA or IgG (100ng/well, Alpha Diagnostics) or gp120 (200ng/well, ABL), then blocked for two hours at room temperature with 1% BSA blocking solution (KPL). Plates were loaded with samples and total Ig standards. Following incubation at 37°C for one hour, the plates were washed 5 times with wash buffer (KPL), and α-Mon IgA-peroxidase or α-Mon IgG-peroxidase (Alpha Diagnostics) diluted 1 to 10,000 in blocking buffer was added and incubated for 1 hour at room temperature. After washing 5 times again, peroxidase substrate was added (KPL), and after 15 minutes Phosphoric acid (1M) was added prior to reading the plate's absorbance at 450 nm. Results are expressed in the concentrations listed based upon the standard curves generated.



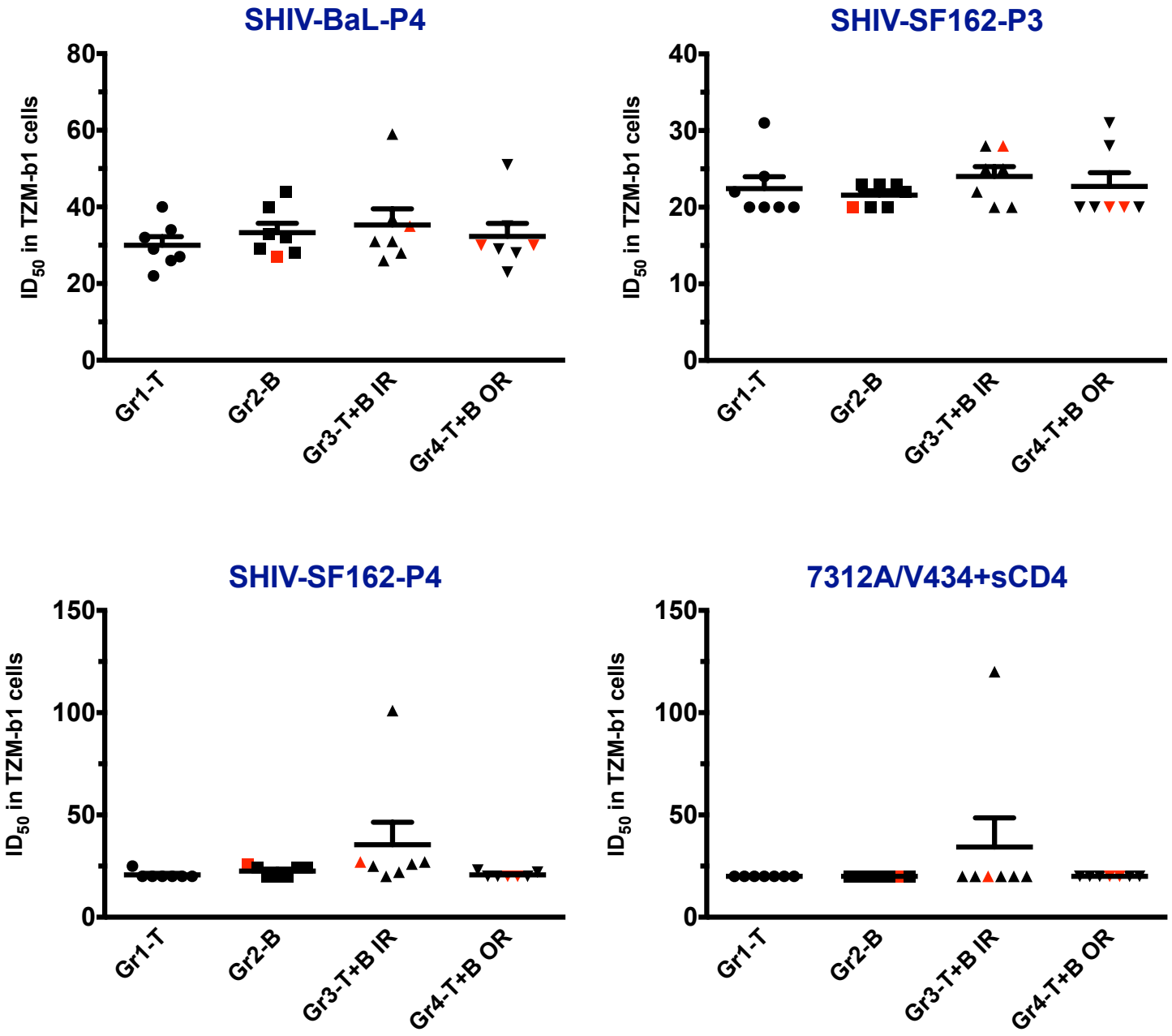
Supplementary Figure1. The distribution of Eudragit FS-30D single-coated BSA-FITC and double-coated BSA-Alexa 647 particles in the gut of the macaques. Eudragit FS-30D single-coated BSA-FITC and double-coated BSA-Alexa 647 particleswere given orally to the macaque, and followed by necropsy after 24hrs. Single cell suspensions from duodenum, jejunum, ileum, caecum, ascending, transverse, descending colons, and rectum were collected. FACS were run to identify the florescent-labeled particles in live cells.



Supplementary Figure2. Anti-Env antibody responses in the plasma and rectal mucosa in the first study.

A) Binding antibody responses against FLSC, gp120 and CD4 were measured in plasma samples collected before and 2w post last vaccination. The red ones indicated the protected animals. Since HIV-1 envelop did not include in the vaccine received by group1 animals, the post-vaccination levels of FLSC and gp120 titers in this group also served as baseline levels, as the animals from group 5, and naïve group.

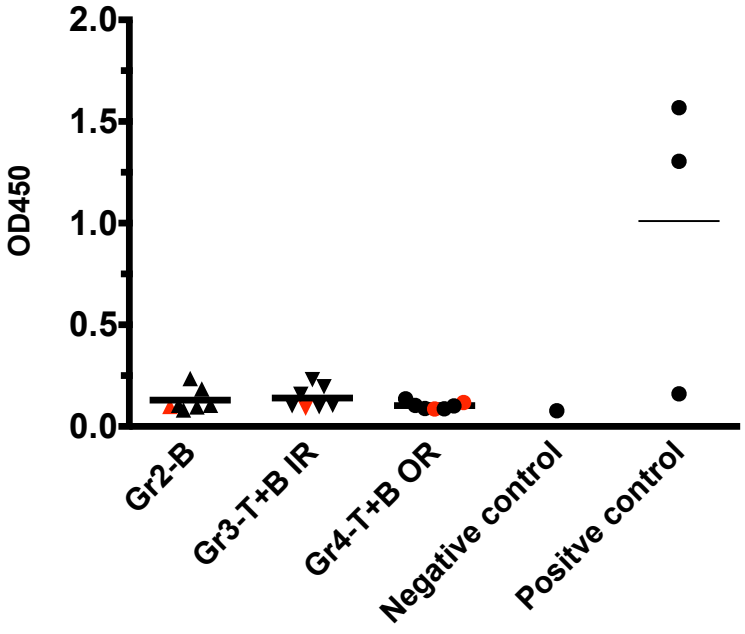
B

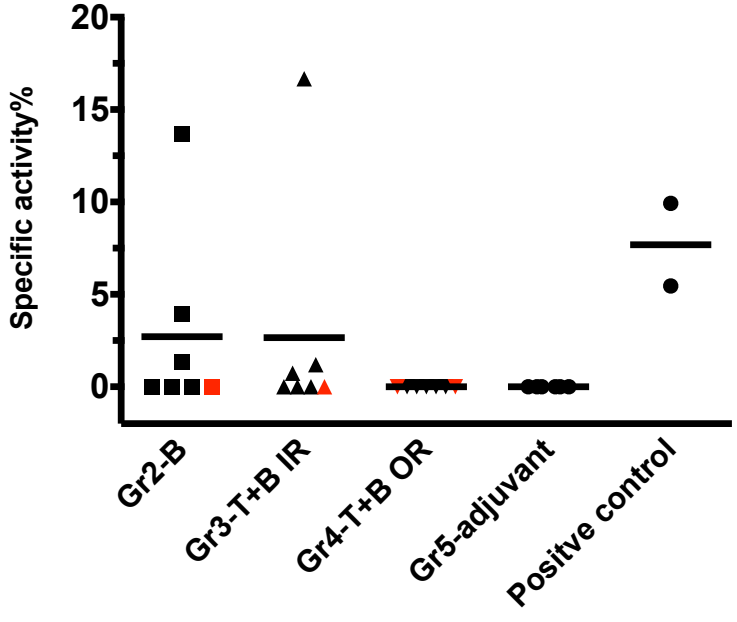


Supplementary Figure2. Anti-Env antibody responses in the plasma and rectal mucosa in the first study.

B) Neutralizing antibody responses against SHIV were measured in plasma samples collected 2w post last vaccination. The red ones indicated the protected animals.

C

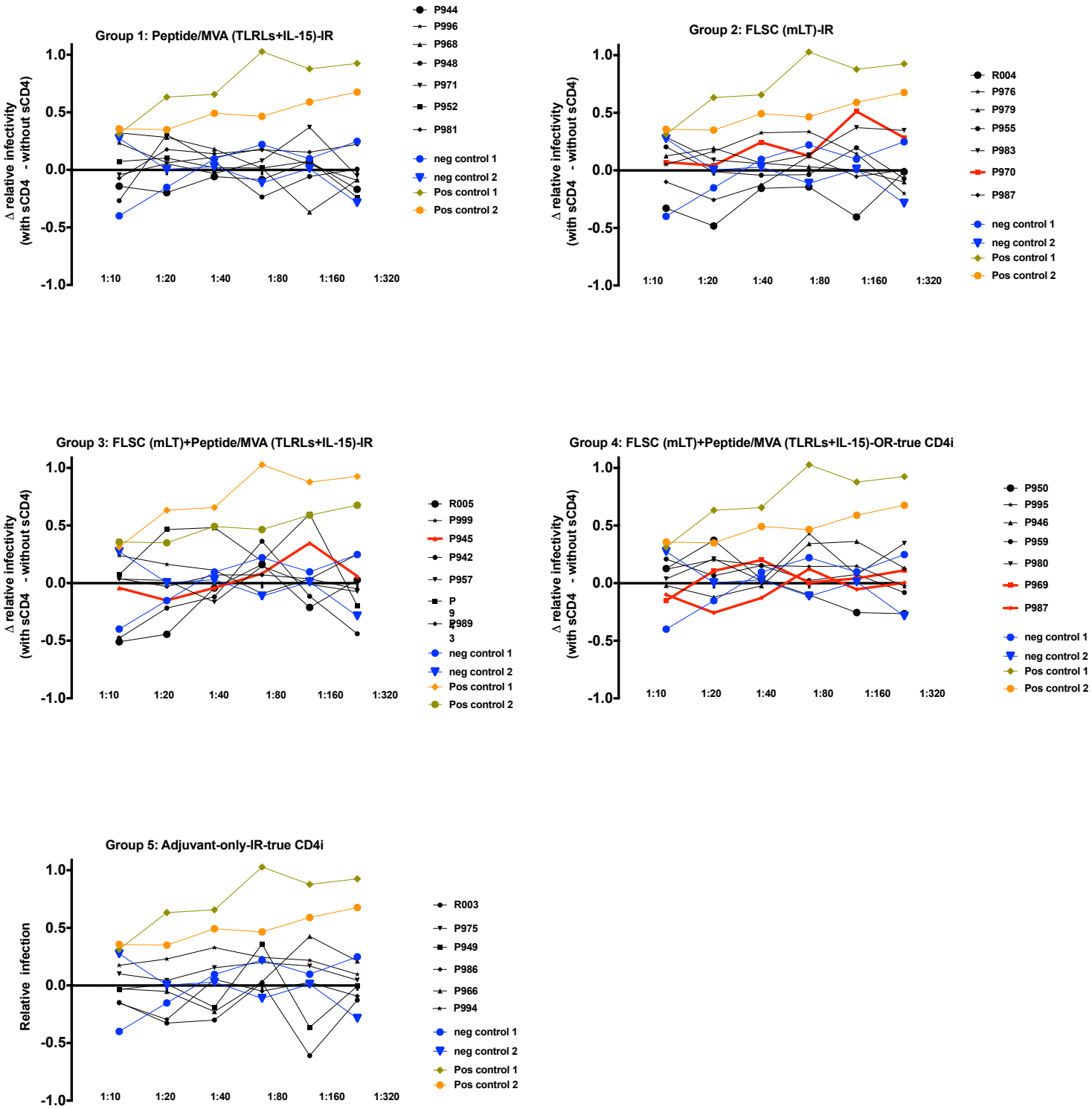




Supplementary Figure2. Anti-Env antibody responses in the plasma and rectal mucosa in the first study.

C) Mucosal antibody responses against FLSC antibody were measured in rectal washes and mesenteric LNs collected 4w post last vaccination. The red ones indicated the protected animals.

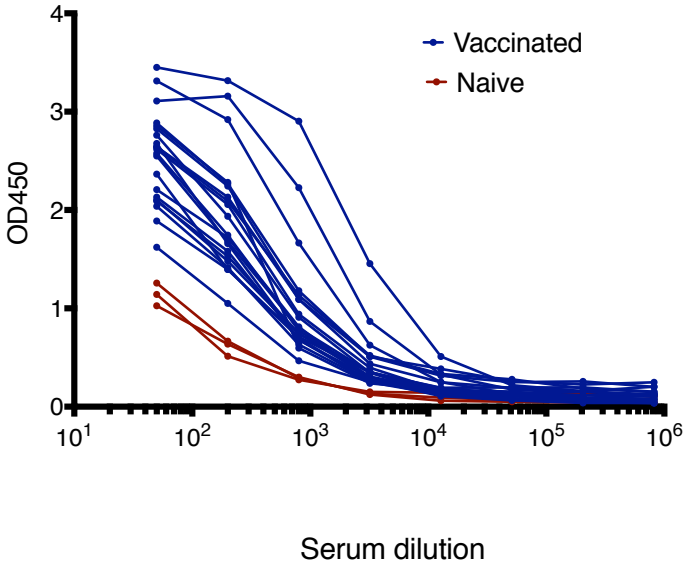
D



Supplementary Figure2. Anti-Env antibody responses in the plasma and rectal mucosa in the first study.

D). CD4-inducible antibody responses against FLSC were measured in plasma samples collected 2w post last vaccination. The red ones indicated the protected animals. Since all samples were measured in one experiment (with two negative and positive controls), the data from these controls are repeated in each panel.

A



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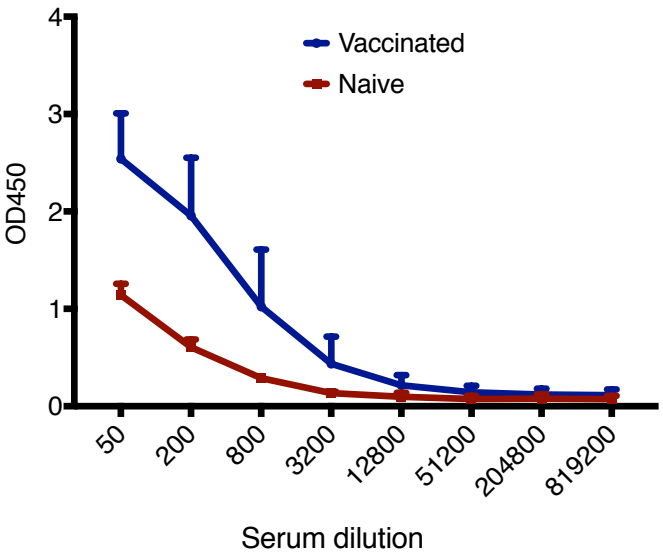

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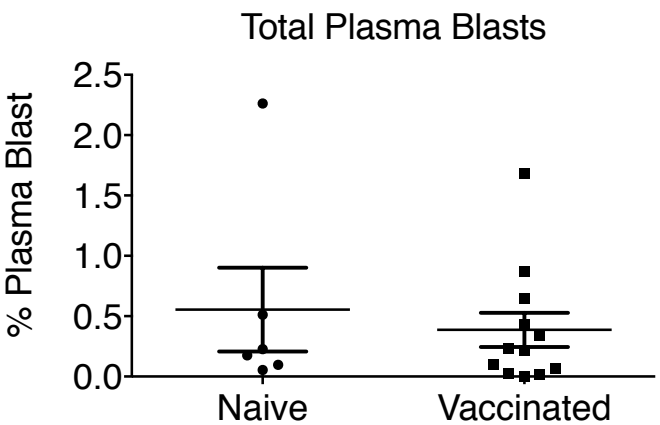
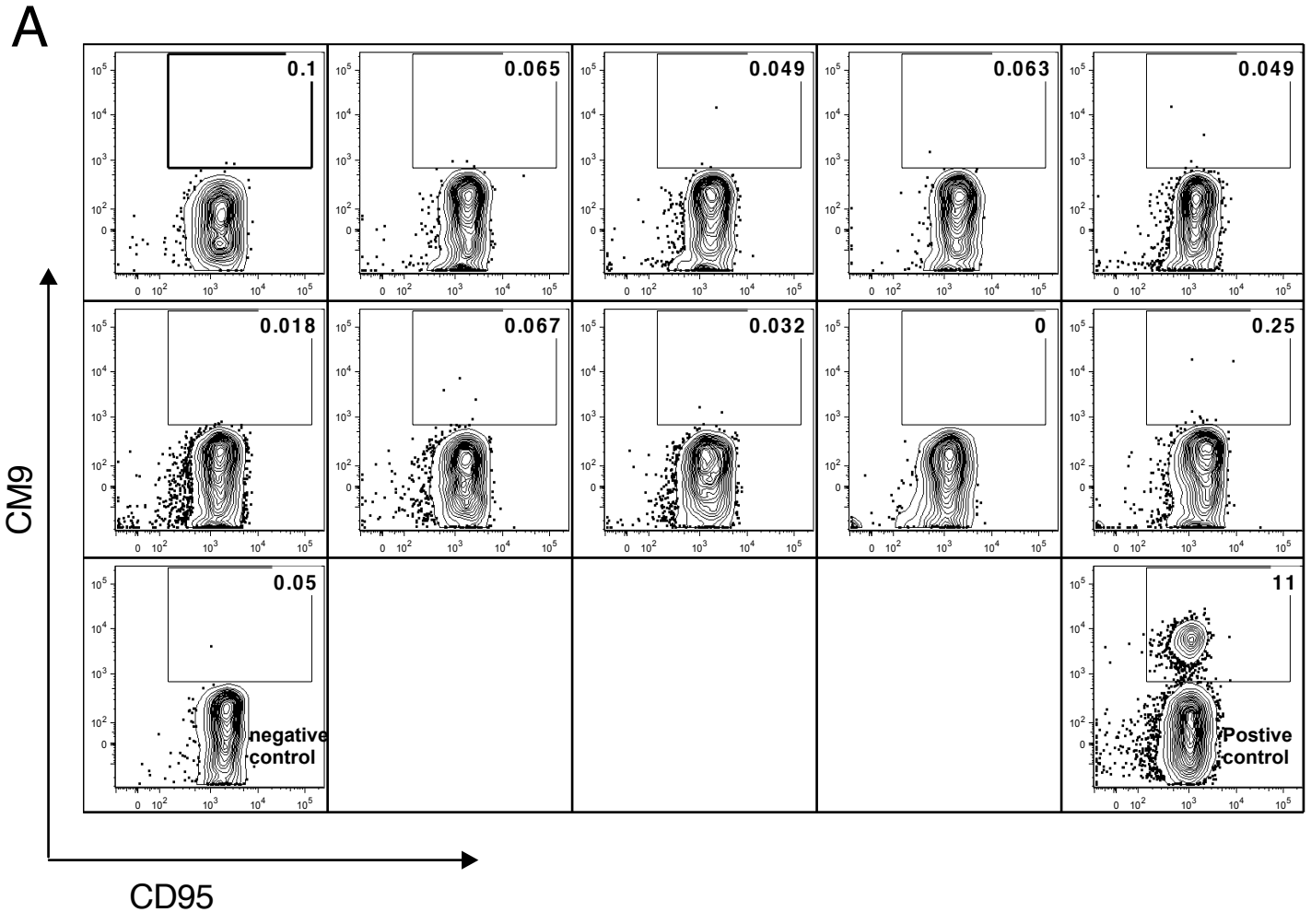
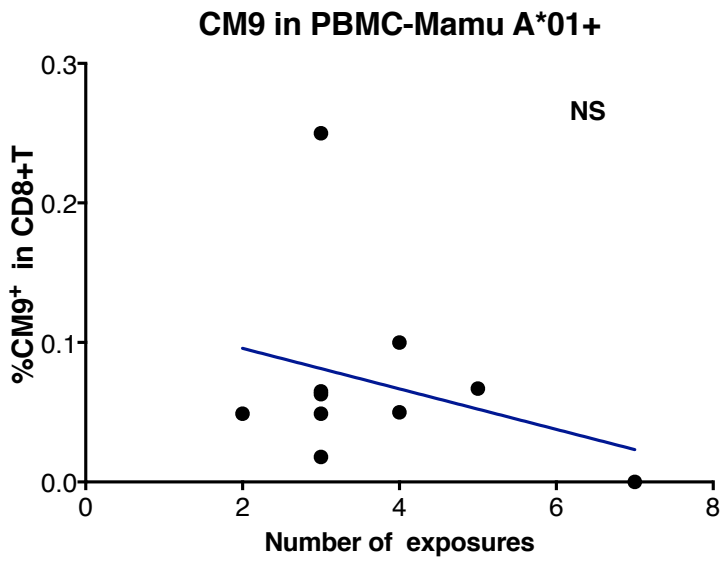
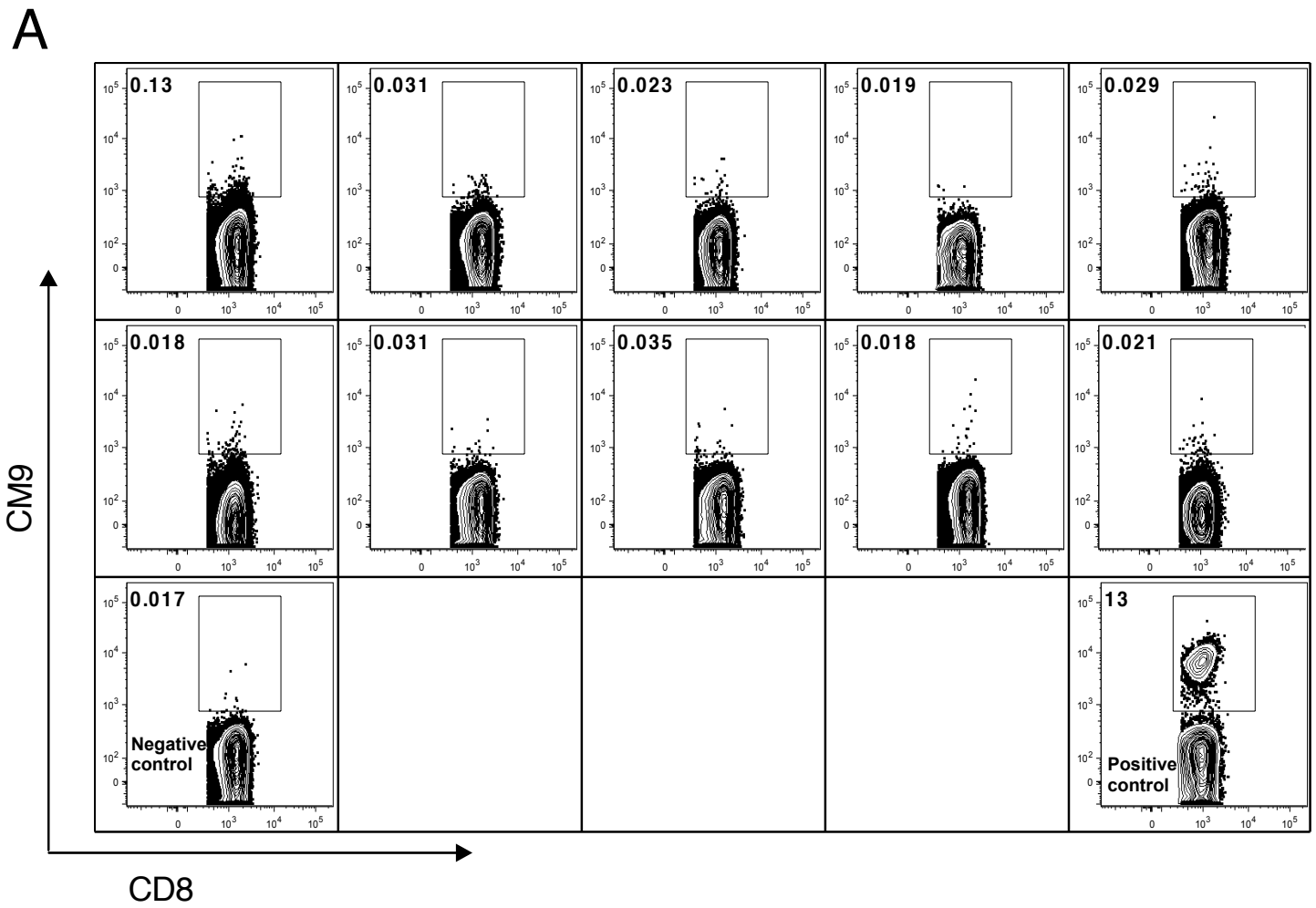


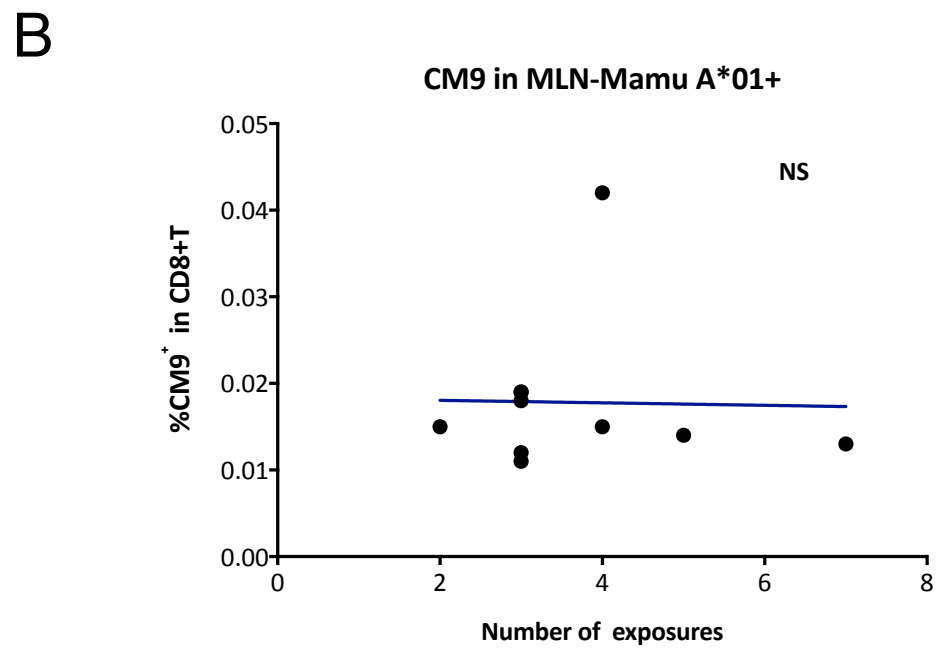
Supplementary Figure 3. The humoral responses in the third cohort. A-B). Anti-mLT IgG responses were induced in the plasma of the vaccinated (n=21) animals 2w post last vaccination. The naive animals (n=3) had low level of anti-mLT antibody responses. Mean±SDs are shown in B. C). The frequencies of the total plasma blast cells in the rectal pinches of the naive and vaccinated animals were compared.



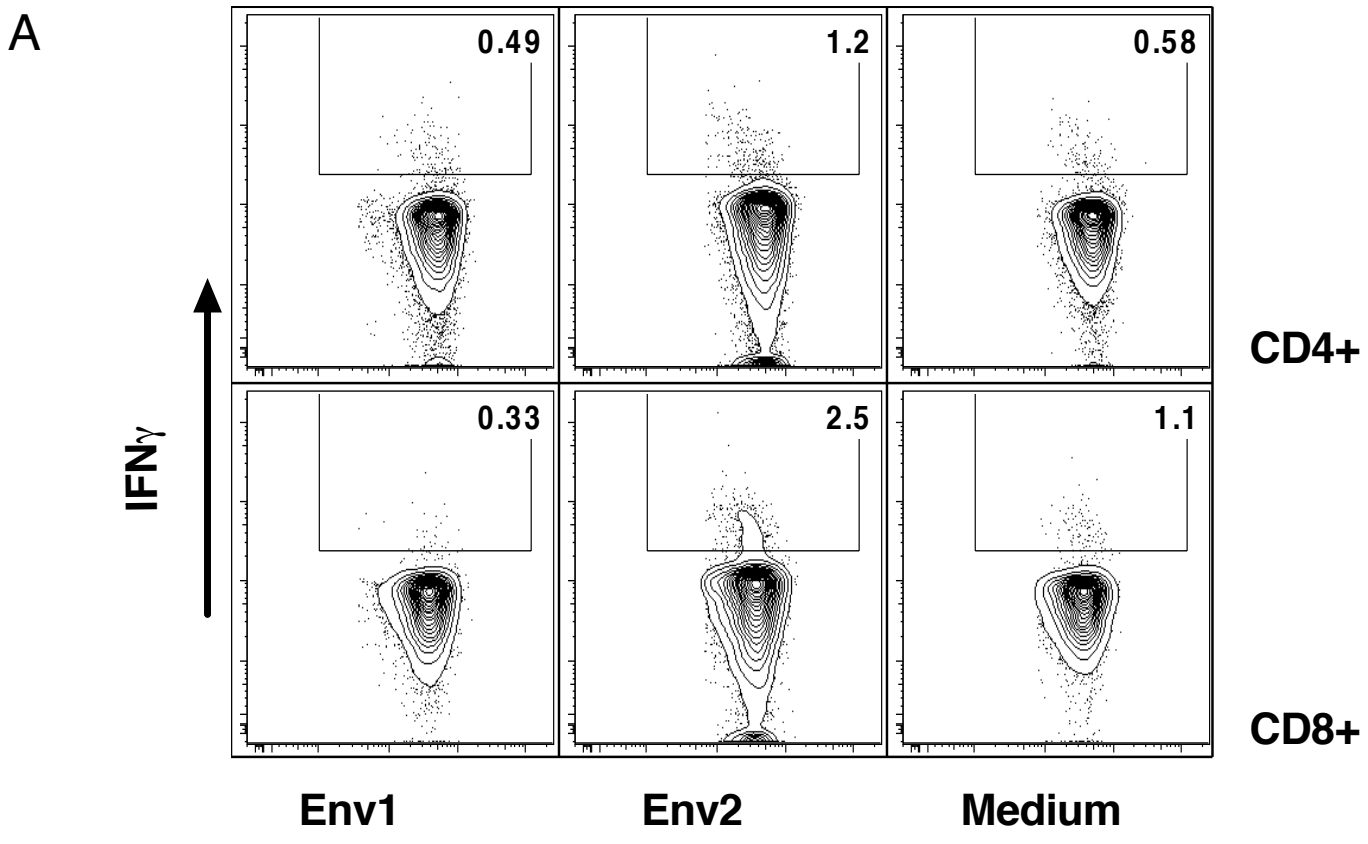


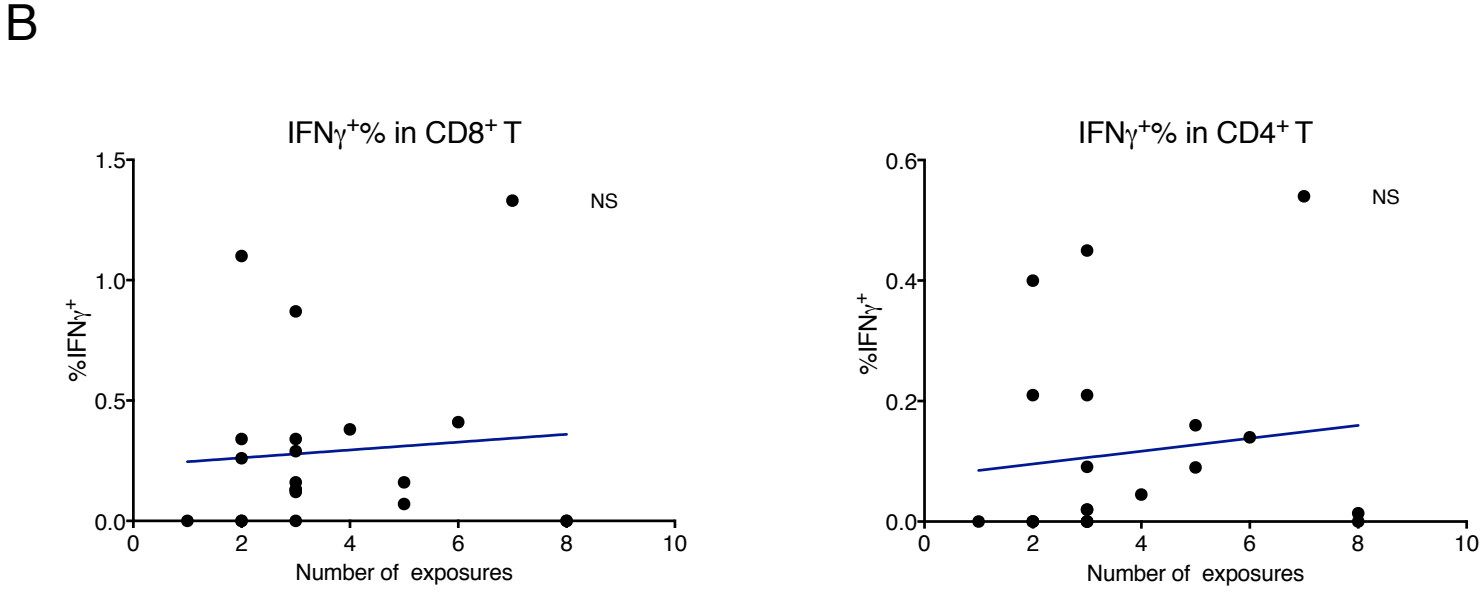
Supplementary Figure 4. The $CM9^{+}CD8^{+}$T cell responses in the PBMCs of the 10 vaccinated Mamu A*01-postive animals in the third study. A) The $CM9^{+}CD8^{+}$T cell responses in the PBMC; B) the responses did not correlate with number of exposures for the animals to be infected. PBMC samples were collected 2-week post last vaccination. Spearman's tests were used to calculate the correlations.



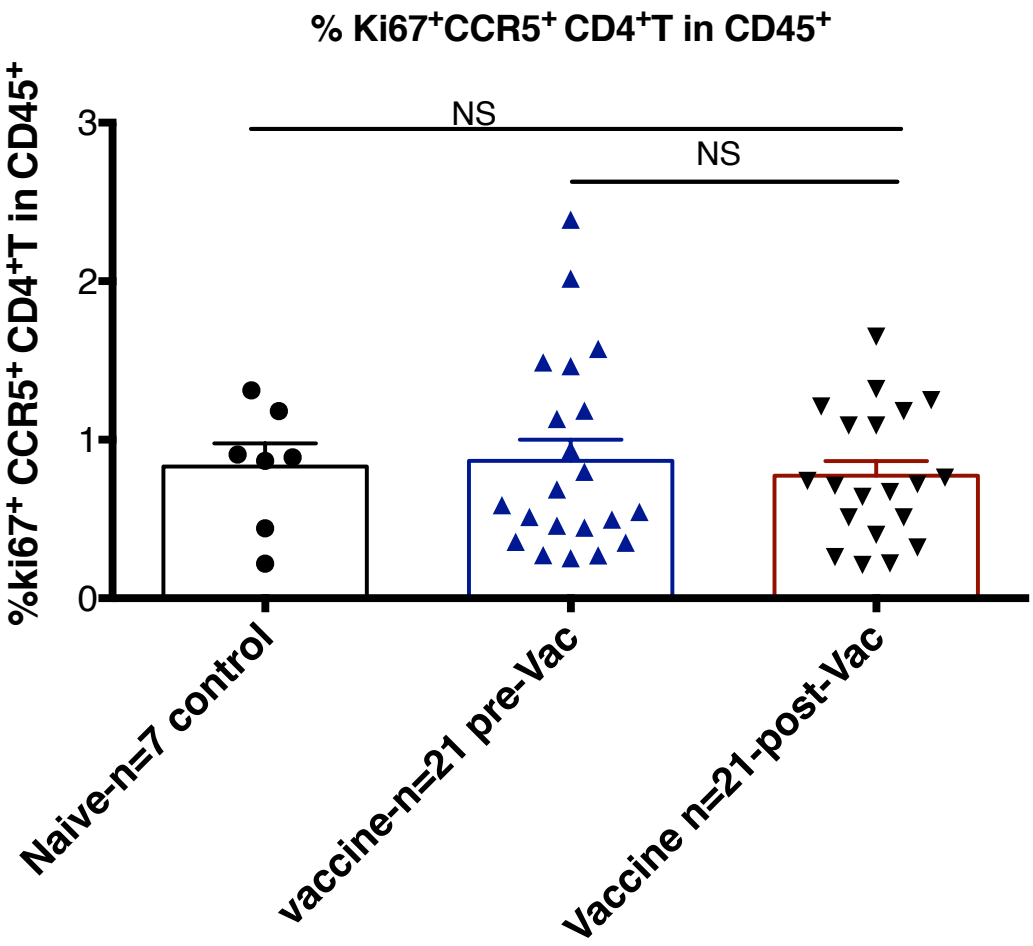


Supplementary Figure 5. The $CM9^{+}CD8^{+}$T cell responses in the mesenteric LNs of the 10 vaccinated Mamu A*01-postive animals in the third study. A) The $CM9^{+}CD8^{+}$T cell responses in the mesenteric LNs; B) the responses did not correlate with number of exposures for the animals to be infected. mesenteric LN samples were collected 4-week post last vaccination. Spearman's tests were used to calculate the correlations.

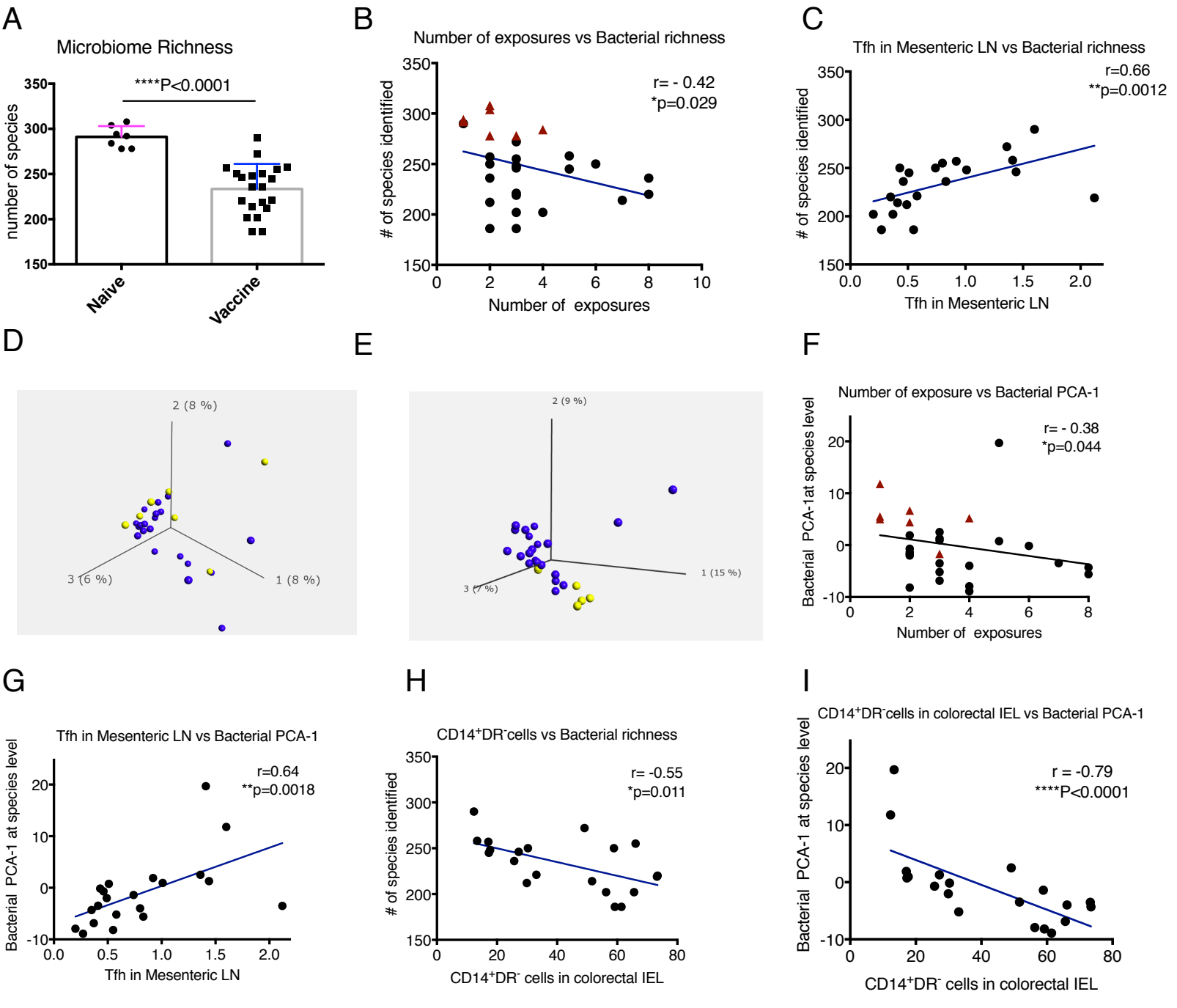




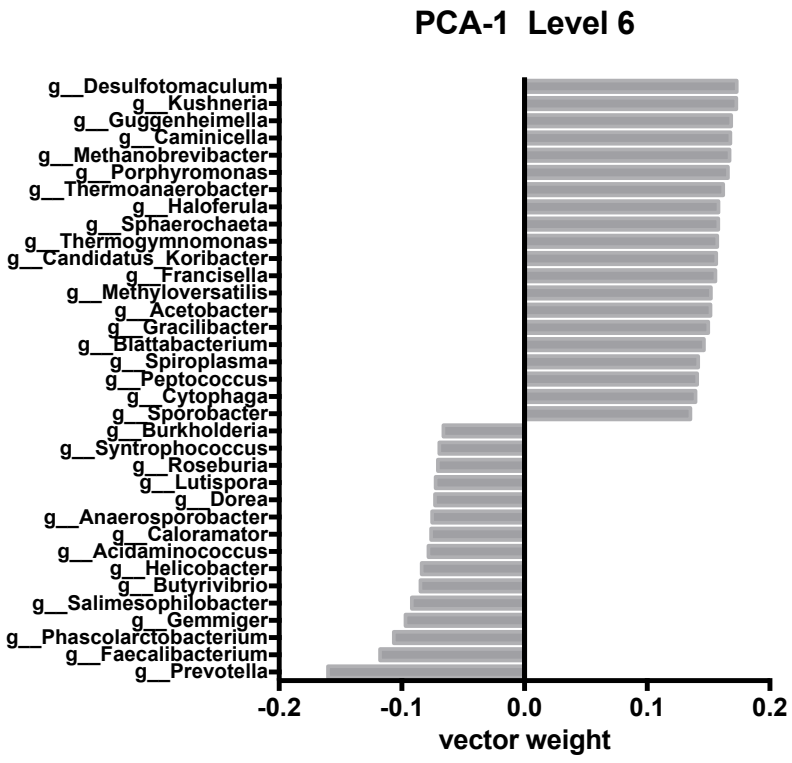
Supplementary Figure 6. Env-specific intracellular staining of IFNγ$^+$ T cells in the MLN of macaques 4 weeks post last vaccination in the third study. A). Gating strategies for the Env-specific CD4+ and CD8+ T cells. 15-mer HIV bal envelope peptide pools (11aa overlapping) were split into Env1 and Env2 to stimulate the cells. B). The magnitude of Env-specific T cell responses did not correlate with delay of viral acquisition. Spearman's *r* correlations were used.



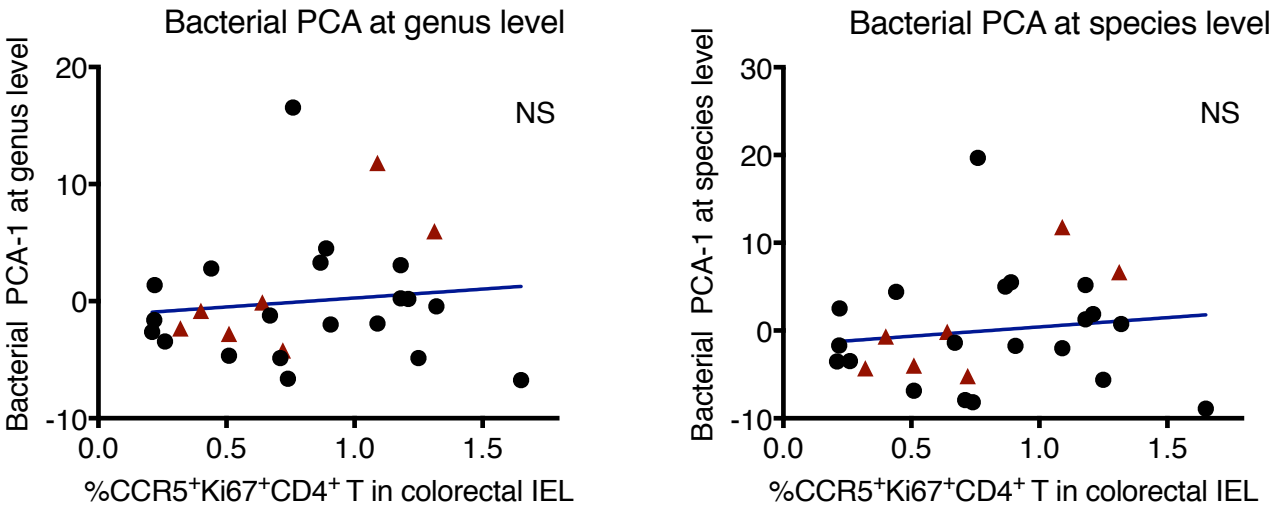
Supplementary Figure 7. Mucosal vaccination did not change the viral target cells in the rectal mucosa in the third study. The frequency of $Ki67^{+}CCR5^{+}CD4^{+}$ viral target cells in the rectal intraepithelial lymphocytes did not significantly differ from those of pre-vaccination, and naïve controls. Mann-Whitney rank sum tests were used for comparisons.



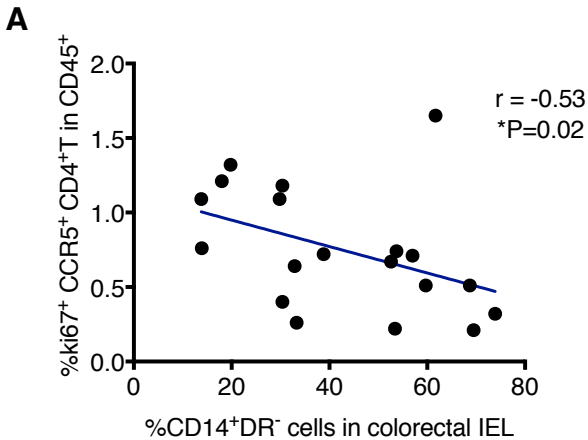
Supplementary Figure8. Gut microbiome at species level correlated with viral exposure, Tfh in MLN, and newly migrated monocytes in the IEL of the colorectal tissues in the third cohort. A) The diversity of the gut microbiome at the species level was decreased after vaccination. Mann-Whitney rank sum test was used for comparison. B-C) Bacterial richness inversely correlated with viral acquisition (B), and positively correlated with Tfh cells in the MLN (C). D-E) The Principal component analysis (PCA) plot of the gut microbiome at the species level one month before vaccination(D) and 7-week after the last vaccination(E). The blue dots indicate vaccinated animals, while the yellow ones indicate the naïve animals. F-G). The PCA-1 of the gut microbiome at species level was exported for each animal. The bacterial PCA-1 showed associations with number of viral exposure (F), Tfh in the mesenteric LN(G). H-I) $CD14^{+}DR^{-}$cells in the IEL of the colorectal tissues correlated with bacterial richness (H) and bacterial PCA-1 at species level (I). Spearman's tests were used to calculate the *r* and *P* value of the correlations.

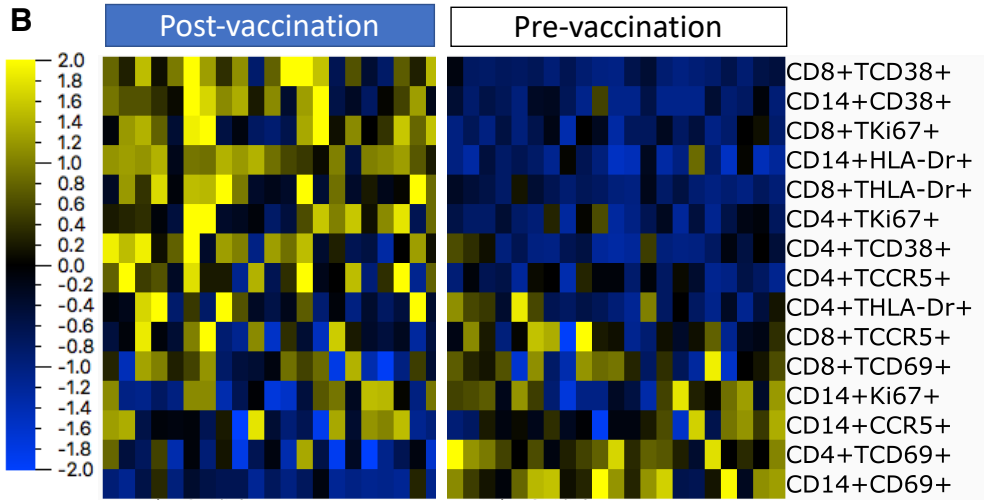


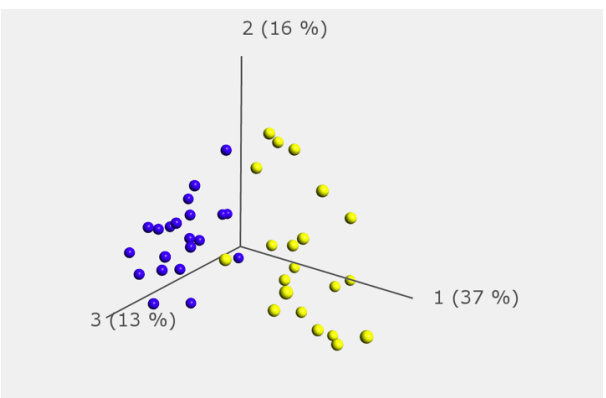
Supplementary Figure9. Gut microbiome analysis of the macaques in the third study. The top genera composition of PCA-1 at genus level after vaccination.



Supplementary Figure10. The bacterial PCA-1 did not correlate with viral target cells in the rectal mucosa in the third study. Spearman's *r* correlations were shown.







D

	Pre-vac			Post-Vac		
Gut microbiome	Immune-markers	P	R	Immune-markers	P	R
PCA-1	NA			CCR5+ on CD14+cells	0.002	-0.64
	NA			CD69+ on CD8+T	0.047	0.44
	NA			CD69+ on CD14+cells	0.047	0.44
PCA-2	Ki67+ on CD8+T	0.03	-0.46	CCR5+ on CD8+T	0.03	-0.47
	NA			CD38+ on CD8+T	0.03	-0.47
	NA			CD38+ on CD4+T	0.04	-0.45
PCA-3	HLA-DR+ on CD4+T	0.02	0.50	CD69+ on CD8+T	0.04	-0.45
	HLA-DR+ on CD8+T	0.04	0.45	Ki67+ on CD14+cells	0.049	0.43

Supplementary Figure 11. Mucosal vaccination did not change the viral target cells in the rectal mucosa in the third study. However, the other immune markers in the rectal mucosa were differnt after vaccination. A).The frequency of $Ki67^+CCR5^+CD4^+$ viral target cells in the rectal intraepithelial lymphocytes inversly correlated with CD14+DR- cells. B, C). the heatmap and the PCA plot of other immune actiation markers (CCR5, KI67, CD69, CD38, and DR) on CD4+T, CD8+T and CD14+ monocytes in the rectal mucosa. D).The correlations between PCAs of gut microbiome and the immune activation markers in the colorectal IEL pre and post-vaccination.

Supplemental Table 1. The basic information of rhesus macaques included in the first study

		ID#	*Animal ID*	*Sex*	*DOB*	*Weight*	*A01*	*A02*	*B08*	*B17*
Gr1: T cell vaccine	1	P981	DCFW	M	1/1/08	3.3	+	-	-	-
Route: IR	2	P948	JJ7	F	1/1/08	4.3	+	-	-	-
	3	P952	JZW	F	1/1/08	3.3	+	?	?	?
	4	P971	JF3	F	1/1/08	3.5	-	+	-	-
	5	P968	JD4	F	6/1/08	4.1	-	+	-	-
	6	P944	DCMP	M	1/1/08	4.2	-	+	-	-
	7	P996	JMZ	F	3/25/08	4.6	-	+	-	-
					MEAN WT	**3.9**				
Gr2: B cell vaccine	1	P970	JG1A	F	1/1/08	3.8	+	-	-	+
Route: IR	2	P955	JR9	F	1/1/08	3.7	+	-	-	-
	3	R004	DBPW	M	1/1/06	6.5	+	-	-	-
	4	P976	DE0G	M	1/1/08	3.2	-	+	-	-
	5	P990	JK6	F	4/14/08	4.3	-	+	-	-
	6	P979	DCBH	F	4/22/08	4.0	-	+	-	-
	7	P983	DCMX	M	1/1/08	3.7	-	+	-	-
					MEAN WT	**4.2**				
Gr3: T+B cell vaccine	1	P989	J1J	F	1/1/08	3.8	+	-	-	+
Route: IR	2	P999	J1Z	F	1/1/08	3.9	+	+	-	-
	3	R005	DC89	M	7/14/06	5.1	+	-	-	-
	4	P943	J98	F	4/3/08	4.6	-	+	-	-
	5	P942	JA0	F	4/13/08	3.7	-	+	-	-
	6	P957	JA1	F	1/1/08	3.4	-	+	-	-
	7	P945	DCMJ	M	3/30/08	3.4	-	+	-	-
					MEAN WT	**4.0**				
Gr4:T+B cell vaccine	1	P959	J47	F	3/6/08	2.9	+	-	-	-
Route: IR+Orally	2	P969	JB1	F	4/19/08	3.5	+	-	-	-
	3	P980	DCGP	M	5/15/08	3.7	+	+	-	-
	4	P987	DCAL	F	4/22/08	4.2	-	+	-	-
	5	P950	J68	F	1/1/07	4.2	-	+	-	-
	6	P995	G0A	F	1/1/07	3.9	-	+	-	-
	7	P946	JIL	F	1/1/08	4.7	-	+	-	-
					MEAN WT	**3.9**				
Gr5: adjuvant only	1	P975	DCKC	M	1/1/08	3.6	+	+	-	-
Route: IR	2	P949	JJ1	F	4/1/08	4.1	+	-	-	-
	3	P966	DCGZ	M	4/1/08	3.6	+	?	?	?
	4	P986	DCAW	M	6/1/08	5.0	-	+	-	-
	5	R003	DCHZ	M	4/21/08	3.6	-	+	-	-
	6	P994	GRC	F	1/1/07	4.2	-	+	-	-
					MEAN WT	**4.0**				
Gr6: Niave	1	R103	R103	M	4/1/08	4.1	+	-	-	-
	2	P988	J1L	F	1/1/08	3.7	+	?	?	?
	3	P953	JZZ	F	6/1/08	3.2	+	?	?	?
	4	P978	DCEL	M	6/1/08	4.7	-	+	-	-
					MEAN WT	**4.0**				

Supplementary Table 2: Design of the first vaccine study (high-dose challenge): group information*

Groups	Reagents	Vaccination Route	NHP #
1: T cell vaccine	T cell vaccine components Primed twice with: DOTAP (100 μL) mixed with peptides**+IL15(300 μg) +500 μg per dose of D-type CpG oligodeoxynucleotide (TLR 9 agonist), 10 μg per dose of MALP2 (TLR 2/6 agonist), 1 mg per dose of PolyI:C (In Vivogen) (TLR 3 agonist) Followed by boosting twice with: MVA-SIVmac239 Gag, pol, env and Rev, Tat, Nef (5 × 108/immunization for each)	IR	7
2: B cell vaccine	B cell components Primed twice +boosted twice with: FLSC-gp120(100μg per dose) + mLT(100μg per dose) in DOTAP (100 μL)	IR	7
3: T+B cell vaccine	T+B cell vaccine components	IR	7
4: T+B cell vaccine	T cell vaccine components +B cell vaccine components	IR PO	7
Control	Adjuvant control: IL-15+TLRLs + mLT	IR	6
Control	Concurrent naïve control		12
Control	Adenovirus vector control used as for mock control in other arm of the large collaborative study		4
Control	Historical naïve (from virus batch titration)		7

*: immunization schedule was shown in Figure 1.

**: a mixture of SIV/HIV peptides was listed in reference 3.

Supplementary Table 3: Summary of humoral immune responses measured in the first study*

	Humoral immune responses measured	Results	Raw data
1	Binding antibodies in plasma	very low binding antibody titers to gp120, FLSC, and CD4	Supplementary Figure 2a
2	Neutralizing antibodies against SHIV in plasma	Very low	Supplementary Figure 2b
3	Mucosal antibodies (rectal swab and mesenteric LN)	No detectable mucosal IgG and IgA antibodies against HIV gp120	Supplementary Figure 2c
4	CD4-inducible antibodies in plasma	Very low	Supplementary Figure 2d
5	ADCC in plasma	Not detectable	
6	B cell ELISPOTs in mesenteric LN	Not detectable	

* The plasma, rectal swab and mesenteric LN samples were collected 2-4weeks post the last vaccination.

Supplemental Table 4. The basic information of rhesus macaques included in the third cohort study

		Animal ID	Gender	Date Birth	weight	A01	B08	B17	B29
Vaccine	1	MLP	Female	1/1/11	4.20	+	-	-	-
	2	A10X010	Female	5/6/10	5.06	+	-	-	-
	3	ML9	Female	1/1/11	4.60	+	-	-	-
	4	MAB	Female	1/1/11	4.53	+	-	-	-
	5	DEZT	Male	5/1/11	3.40	+	-	-	-
	6	DF07	Male	1/1/11	3.40	+	-	-	-
	7	DFMZ	Male	1/1/11	3.20	+	-	-	-
	8	DX7E	Female	6/2/10	5.23	-	-	-	-
	9	GB7P	Female	5/31/10	4.53	-	-	-	-
	10	GB7L	Female	7/28/10	4.29	-	-	-	-
	11	DX7L	Female	5/11/10	4.37	-	-	-	-
	12	DX8T	Female	5/19/10	4.09	-	-	-	-
	13	GB5V	Female	5/31/10	3.75	-	-	-	-
	14	H162	Female	5/13/11	3.60	-	-	-	-
	15	DEEZ	Male	4/25/10	5.90	+	-	-	
	17	DEK3	Male	3/30/10	5.14	+	-	-	
	20	R298	Male	7/25/08	8.98	+	-	-	
	24	821/JHM	Female	4/2/09	4.61	-	-	-	-
	25	823/KLK	Female	1/1/09	5.01	-	+	-	-
	27	825/MG6	Female	1/1/10	5.79	-	-	-	-
	28	822/GB5	Female	5/23/09	4.49	-	-	-	-
Naïve	16	DEK2	Male	5/1/10	7.18	+	-	-	
	18	DEM7	Male	5/1/10	7.22	+	-	-	
	19	R27	Male	4/25/10	6.46	+	-	-	
	21	R51	Female	5/1/08	6.10	+	-	-	
	22	R59	Male	5/2/08	7.62	+	-	-	
	23	861	Male	5/8/08	9.26	+	-	-	
	26	824/KMV	Female	8/1/09	4.82	-	+	-	-

Supplementary Table 5: Summary of humoral immune responses in the third study*

	Humoral immune responses measured	Results	Positive control	Negative/naive control
1	Binding antibodies to gp120, FLSC in plasma(titer/μg total IgG/A)	All negative	Positive	All negative
2	FLSC-IgG in rectal pinch explant culture (titer/μg total IgG/A)	0.10±0	111.10	0.15±0.04
3	FLSC-IgA in rectal pinch explant culture (titer/μg total IgG/A)	0.18±0.02	5.10	0.32±0.08
4	FLSC-IgG in rectal swabs (titer/μg total IgG/A)	1.63±0.19	70.55	1.41±0.28
5	FLSC-IgA in rectal pinch explant culture (titer/μg total IgG/A)	0.24±0.03	3.45	0.29±0.05
6	IgG/A/M to FLSC/gp120-Bal in rectal swab (MFI/μg/ml Ig)	All negative	9379 (pooled HIV+ IgG) 40704 (CH32 IgA-mAb)	All negative

*Plasma samples were collected 2 week post the last vaccination; rectal samples were collected 4 week post the last vaccination. Data are shown as mean ± SEM.

Supplementary table 6: Microbiome data-from one week before viral challenge
The data was provided in the Excel document.

Supplementary Table 7: Flow cytometry analysis panels

SHIV-T cell-ICS	MDSC	Immune activation	CM9-Dextramer	Tfh	Env-B cell
CD3-PE-Cy7	CD3-PE-Cy7	CD3-PE-Cy7	CD3-PE-Cy7	ICOS-PE-Cy7	CD2-Qdot605
CD4-Qdot605	CD4-Qdot605	CD4-Qdot605	CD4-Qdot605	CD4-Qdot605	CD14-Qdot605
CD8-APC-Cy7	HLA-DR-APC-Cy7	CD8-APC-Cy7	CD8-APC-Cy7	CXCR3-APC-Cy7	CD19-PE-Cy5
CD95-PE-Cy5	CD11b-PE-Cy5	HLA-DR-PE-Cy5	CD95-PE-Cy5	CD95-PE-Cy5	CD20-eF650NC
CD28-FITC	Lin-FITC	CD38-FITC	CCR7-FITC	CCR7-FITC	CD21-PE-Cy7
CD14-BV421	CD14-BV421	CD14-BV421		PD-1-BV421	CD27-PerCp-eF710
IFNγ-PE	CD33-PE	CCR5-PE	CM9-Dextramer	BCL6-PE	CD138-PE
Ki67-APC	Ki67-APC	Ki67-APC	CD28-APC	CXCR5-APC	Biotinylated SIV_{mac251}ENV
TNFα-Alexa700	CD15-Alexa700	CD45-Alexa700	CD45-Alexa700	CD3-Alexa700	IgD-Texas Red
IL-17A-BV711	CD69-Percp-Cy5.5	CD69-Percp-Cy5.5			IgG-APC-Cy7
IL-2-BV785					Ki67-Alexa700
Yellow viability	Yellow viability	Yellow viability	Yellow viability	Yellow viability	IRF-4-eFluor660/FITC
					HLA-DR-Qdot800
					Aqua viability