Supporting Information

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SI Materials and Methods

Vectors. All recombinant adenoviral vectors used in this study carried an identical e*gfp* gene under the control of a cytomegalovirus (CMV) promoter. The first-generation Ad5-eGFP (E1-, E3-deleted) was purchased from Avior Therapeutics Inc. Ad11-eGFP (E1-deleted) was constructed as described previously (1). Second generation Ad5-eGFP (E1-, E3-, polymerase-, pTP-deleted) was a kind gift from Professor Andrea Amalfitano (Michigan State University, Michigan) and was constructed as described previously (2). The latter vector was used to reduce synthesis of adenovirus proteins to a minimum and show that results were not due to newly synthesized adenovirus proteins. Viruses were propagated in cells 911, C7 cells, or 293-Ad11-E1B-55K cells for the first-generation Ad5 vector, for the second-generation Ad5 vector, and the Ad11 vector, respectively. They were purified by two rounds of CsCl banding, desalted using Econo-Pac 10DG columns (Bio-Rad), and resuspended in 10 mM Tris-HCl (pH 8), 2 mM $MgCl_2$, and 10% glycerol and stored at −80 °C.

Neutralization Assays. Ad5 and Ad11 antibody titers in serum from 20 healthy donors were quantified as previously described in (3). In brief, 2-fold serial dilutions of serum (1:20, 1:40, 1:80. 1:160, 1:320, and 1:640) were incubated with Ad5 or Ad11 vectors (1,000 vp/cell) for 30 min at room temperature and then added to confluent A549 cells. Cells were cultured for 48 h in Dulbecco's Modified Eagles medium (DMEM) containing 10% FCS, 1% penicillin/streptomycin, and 1% L-glutamine (all from Sigma-Aldrich). eGFP expression was then analyzed by fluorescent activated cell sorter (FACS) analysis. Antibody titers were determined as the highest serum dilution that inhibited 90% cell infection compared to controls.

Blood Samples. Buffy coats were obtained from the North London Blood Transfusion Service. Blood was also obtained from 20 healthy volunteers. PBMCs were isolated by centrifugation over Ficoll-Histopaque and then separated into a low-density fraction enriched for monocytes and a high-density fraction enriched in T lymphocytes by centrifuging over a 50% Percoll gradient for 30 min at 300 × *g* (Sigma-Aldrich). Both fractions were aliquoted, frozen, and stored at −140 °C until required. Ethical approval for the study was given by the Riverside Ethical Committee and informed consent was given by donors.

Generation of Dendritic Cells. Human monocyte-derived DCs were generated as previously described in (4) with modifications. In brief we isolated monocytes from Percoll interface cells using CD14 microbeads (Miltenyi Biotec), which were then cultured in serum-free DC base media (StemX vivo, R&D Systems) for 7 days. Cells were cultured with IL-4 (100 ng/mL) and GM-CSF (100 ng/mL, both from R&D Systems) every other day. The resulting immature DCs were washed twice and re-suspended in RPMI-1640 supplemented with 5% human AB serum (both from Sigma-Aldrich).

CFSE Staining. Percoll pellet cells, enriched for T cells, were thawed and rested overnight in the presence of DNase (10 U/mL Roche Diagnostics). Between 10 and 20 × 10^6 live cells were stained with 1 μM CFSE (Invitrogen) as previously described (5). Cells were washed once with RPMI-1640 supplemented with 10% FCS, followed by two washes with RPMI-1640 supplemented with 10% human AB serum (Sigma-Aldrich). Cells were then resuspended at a concentration of 10^6 cells/ml and co-cultured with autologous DCs at a ratio of 10:1. For some experiments, memory CD4 T cells were purified by negative selection using magnetic beads (Miltenyi Miotec) in accordance with the manufacturer's guidelines. The purity of the isolated cells was found to be >90% as determined by FACS staining.

Pulsing and Co-Culture. Proliferative T-cell responses to Ad5 in buffy coats obtained from the Blood Transfusion Service were initially measured by pulsing DCs with Ad5 and co-culturing with autologous lymphocytes for 6 days at different DC:T cell ratios. Tritiated thymidine (0.5 μCi [0.0185 MBq], Amersham plc) was added in the last 16 h of culture. For all experiments DCs were pulsed with 2,500 vp/cell of first- or second-generation Ad5 or Ad11 vectors. As controls, cells were left unpulsed or pulsed with tetanus toxoid (100 ng/mL, Merck Calbiochem), live influenza (strain X31, 20 HA U/mL), or SEB (10 μg/mL, Sigma-Aldrich). DCs were then cultured for 48 h, autologous CFSE-stained lymphocytes added, and culture continued for 3–7 days. In some experiments 3-day DC-T cell cultures were infected with HIV-1_{Bal} (625 pg/mL) (obtained from Program EVA Centre for AIDS Reagents, National Institute for Biological Standards and Control, UK) and incubated for an additional 4–7 days.

Phenotypic Analysis. We used the following monoclonal antibodies conjugated to phycoerythrin (PE), peridinin chlorophyl protein (PerCP), allophycocyanin (APC), phycoerythrin-cyanine (PE-Cy7), or allophycocyanin-cyanine (APC-cy7): CD3, CD4, CD8, CD11c, CD1a, CD195 (CCR5), CD49d (α_4), β_7 (all from BD PharMingen or BD Biosciences), and CCR9 (R&D Systems).

For staining, cells were washed in FACS buffer (PBS supplemented with 2% FCS, 2 mM EDTA, and 1% NaN_3, all from Sigma-Aldrich) then stained them with mAb or their isotype controls on ice for 30 min. Cells were washed and fixed with BD stabilizing fixative (BD Biosciences), then analyzed by six-color flow cytometry on a LSRII (BD Biosciences). The data were analyzed using Flowjo (Tree Star, Inc.).

Intracellular Staining for and Quantitation of HIV-1 p24. Cells were stained with surface antibodies as indicated above for 30 min on ice. They were then washed and fixed with 2% PFA in PBS (Sigma-Aldrich) for 15 min at room temperature. HIV-1 infected cells were identified by intracellular staining for gag-p24 using anti HIV-1 core protein antibody (KC57-RD1, Beckman Coulter) in the presence of 0.1% saponin (Sigma-Aldrich). Cells were incubated for 20 min at room temperature, washed and resuspended in BD stabilizing fixative (BD Biosciences). As a negative control, we stained uninfected cells that were treated and cultured in same manner.

To quantify HIV-1 p24 in cell culture supernatants, we used the VIDAS p24 II kit (bioMerieux) in accordance with the manufacturer's guidelines. Samples were analyzed on a VIDAS automated analyzer.

Intracellular Staining for Cytokines. Dendritic cells were generated as indicated above. Cells were stimulated for 3 h with Ad5, Ad11 (both at 2,500 vp/cell), tetanus toxoid (100 ng/mL, Merck Calbiochem), heat inactivated influenza (30 min at 56 °C, strain X31, 20 HA U/mL), or SEB (10 μg/mL, Sigma-Aldrich). Cells were matured with 1 μg/mL LPS for a further 3 h then

co-cultured with autologous thawed PBMCs for 24 h. Brefeldin A (1:1,000, Sigma-Aldrich) was added to all culture conditions for the last 16 h of co-culture. Cells were harvested and stained with surface antibodies (CD3-APC-cy7, CD4-APC, CD8-PerCP) for 20 min then washed and fixed with 2% PFA in PBS for 10 min at room temperature. Following fixation cells were washed twice with 0.1% saponin (Sigma-Aldrich) in FACS buffer and resuspended in the same buffer in the presence of monoclonal antibodies against IFN-γ, IL-2, and TNF-α conjugated to PE-Cy7, PE, and FITC, respectively (all from BD Biosciences). Cells were incubated for 30 min at room temperature before they were washed and fixed with BD stabilizing fixative (BD Biosciences).

Cells were then acquired on a LSR II (BD Biosciences). A total of 100,000 $CD3^+$ $CD4^+$ T cells were acquired for each sample. Data were analyzed using FlowJo (TreeStar) using a gating strategy previously described in (6). In brief, doublets were removed using an initial gate based on forward scatter area (FSC-A) versus height (FSC-H) plot. Lymphocytes were then identified by a FSC-A versus side scatter (SSC) plot. Subsequently, events were sequentially gated on $CD3^+$ $CD4^+$ $CD8^-$ versus IFN-γ (for CD4 T cells) and $CD3^+$ $CD8^+$ $CD4^-$ versus IFN-γ (for CD8 T cells) to account for receptor downregulation. Following identification of CD4 and CD8 T cells, gates were applied to functional cytokines to identify all of the possible combinations (Fig. S10).

ELISPOT Assays. First, 96-well polyvinylidene difluoride filter plates (Millipore) were coated overnight with anti-IFN-γ monoclonal antibody (10 μg/mL, Mabtech) at 4 °C. Second, DCs were generated as indicated above then incubated for 3 h with Ad5, Ad11 (both at 2,500 vp/cell), or heat inactivated influenza (20 HA U/mL). Cells were matured with LPS (1 μg/mL, Sigma-Aldrich) for a further 3 h, then co-cultured with autologous thawed PBMCs in IFN-γ coated plates in duplicates (2×10^5 PBMCs/well, 1 DC:10 PBMC ratio). Co-cultures were performed for 24 h at 37 °C in RPMI1640 medium supplemented with penicillin/streptomycin (100 IU/mL/100 μg/mL), L-Glutamine (2 mM/mL), and 10% heat inactivated human male serum (all from Sigma-Aldrich). Positive controls consisted of DC-PBMC co-cultures stimulated with 5 μg/mL phytohemagglutinin (PHA, Sigma-Aldrich) whilst negative controls comprised of untreated DCs co-cultured with PBMCs for the same duration. SFCs were then detected according to the manufacturer's guidelines (Mabtech) and color development was performed using the AP conjugate substrate kit (Bio-Rad Laboratories) in accordance with the supplier's protocol. Spots were counted using an automated AID ELISPOT reader (AutoImmun Diagnostika). Donors with at least 20 SFCs per 10^6 PBMCs after background subtraction and a mean spots value of $>$ (mean spots of negative control + 3$\times$ the standard deviation) were considered responding donors.

Statistical Analysis. Results are expressed as mean $\pm$ SD. We used non parametric tests throughout as normality of data distribution could not be tested. We used the Mann-Whitney U test to determine significance between two unpaired groups. The Spearman test was used to determine correlations between two variables. All statistical tests were performed using GraphPad Prism 5 (GraphPad Software). $P < 0.05$ was considered statistically significant.

1. Stone D, et al. (2005) Development and assessment of human adenovirus type 11 as a gene transfer vector. *J Virol* 79:5090–5104.
2. Amalfitano A, et al. (1998) Production and characterization of improved adenovirus vectors with the E1, E2b, and E3 genes deleted. *J Virol* 72:926–933.
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4. Sallusto F, Lanzavecchia A (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* 179:1109–1118.
5. Quah BJ, Warren HS, Parish CR (2007) Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nat Protoc* 2:2049–2056.
6. Betts MR, et al. (2006) HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* 107:4781–4789.

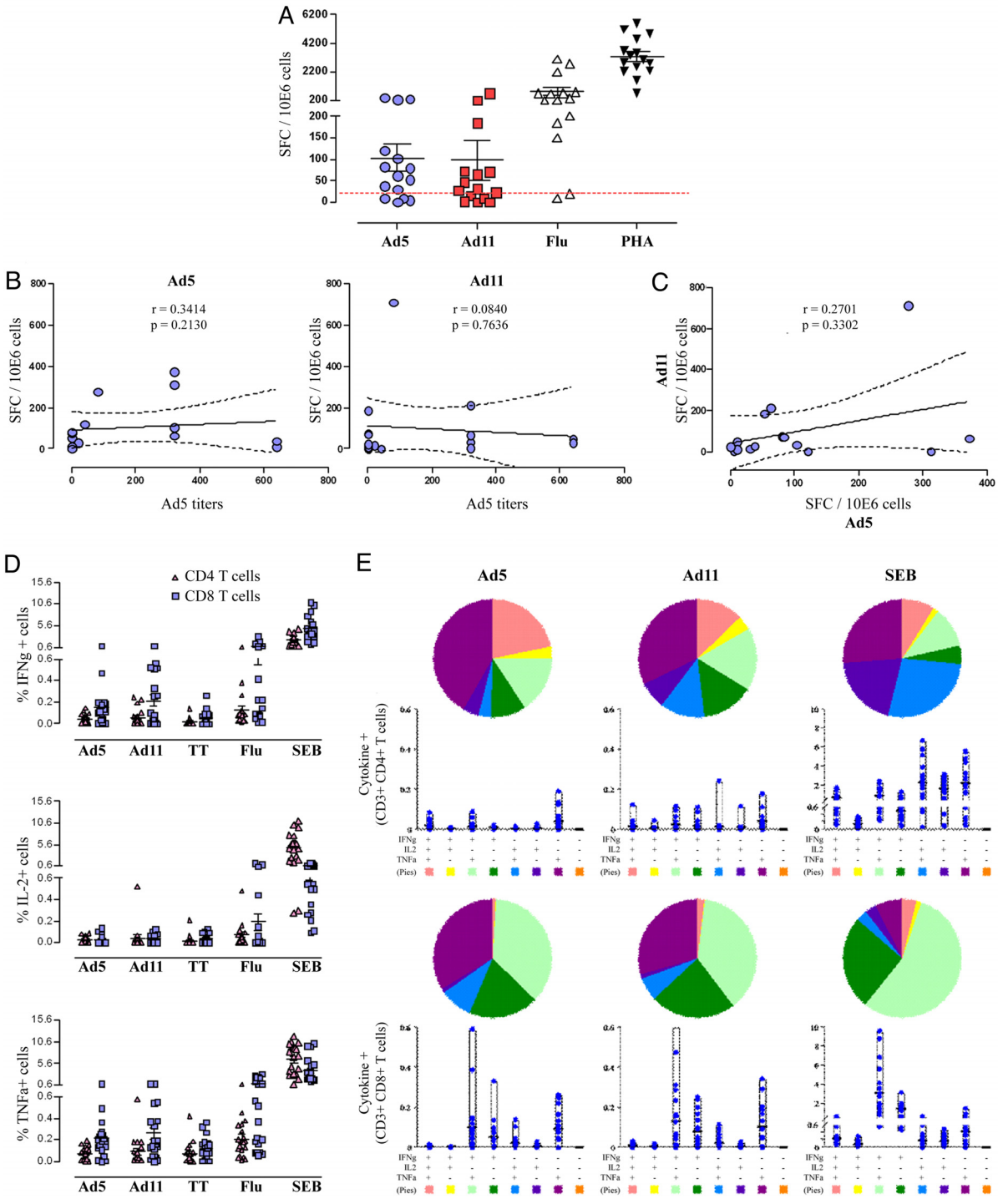

Fig. 1. Ad5 and Ad11 vectors stimulate cytokine production by CD4 and CD8 T cells. (*A*) IFN-γ production by PBMCs ($n = 15$) stimulated with Ad5, Ad11, influenza, or PHA-pulsed DCs. Spot forming cells (SFCs) per 10^6 PBMCs are shown. Dots above red line represent donors with at least 20 SFCs per 10^6 PBMCs after background subtraction and a mean spots value of > (mean spots of negative control + 3× the standard deviation). (*B*) IFN-γ SFCs per 10^6 in response to Ad5 (left graph) and Ad11 (right graph) were plotted against the respective Ad5 titers. *R* and *P* values were obtained using the nonparametric Spearman test. Continued and dotted lines represent the best fit line and 95% confidence intervals respectively. (*C*) SFCs in response to Ad5 (*x* axis) were plotted against those obtained in response to Ad11 (*y* axis). Spearman *R* and *P* values are shown. (*D*) Percentages of IFN-γ (top graph), IL-2 (middle graph), and TNF-α (bottom graph) production by CD4 (triangles) and CD8 (squares) T cells in response to Ad5, Ad11, tetanus toxoid, influenza, and SEB-pulsed DCs as measured by flow cytometry. (*E*) Pie charts represent the mean percentages of CD4 (top panel) and CD8 (bottom panel) T cells expressing single or multiple cytokines in response to stimulation with Ad5 (left column), Ad11 (middle column), or SEB (right column)-pulsed DCs. Bars, the cumulative percentages of the functionally distinct T-cell populations producing all possible combinations of cytokines. Data were corrected for background cytokine secretion in response to unstimulated DCs.

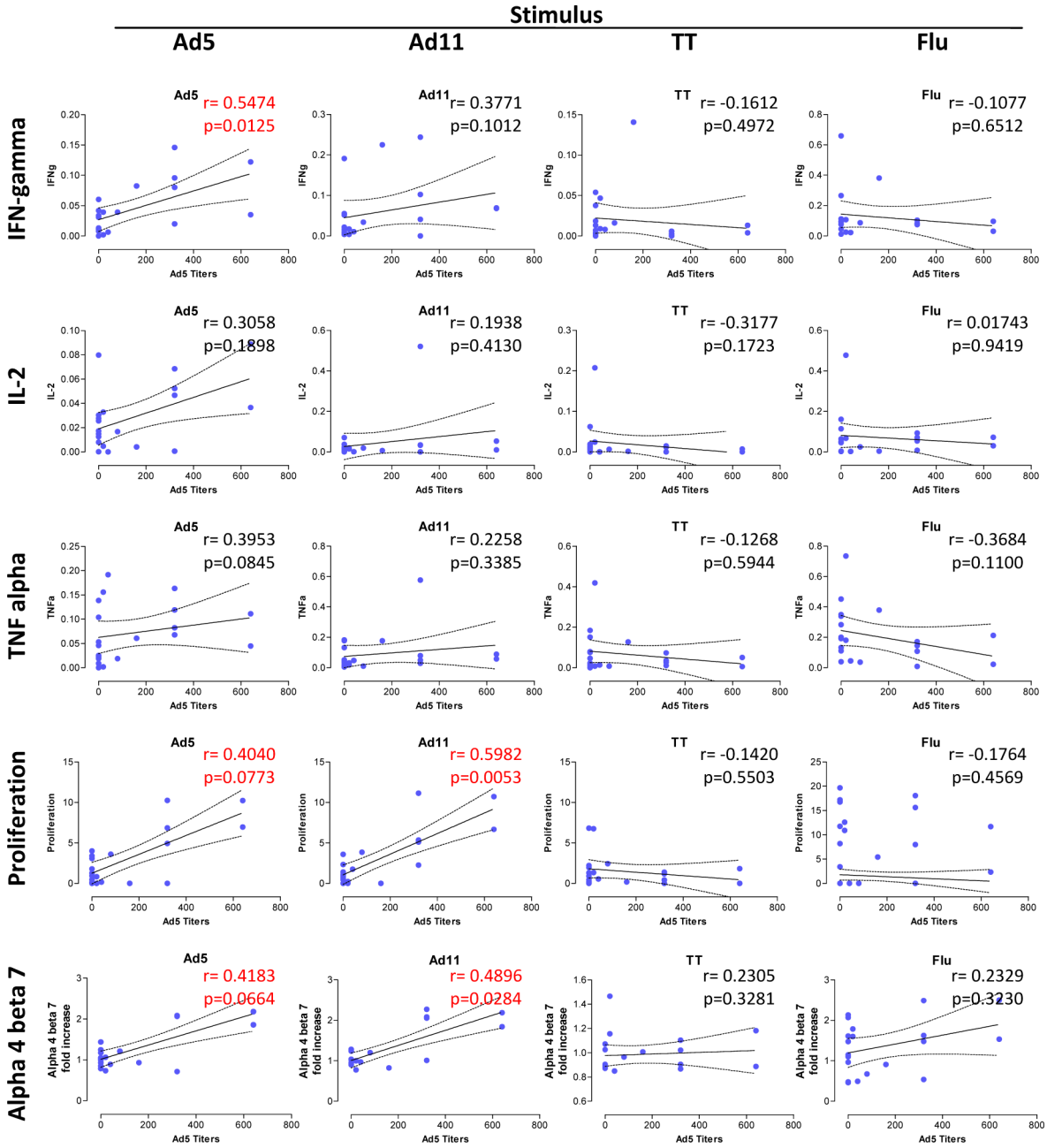

Fig. S2. The first three rows of plots represent the percentages of IFN-γ, IL-2, and TNF-α producing CD4 T-cell in response to (from left to right) Ad5, Ad11, tetanus toxoid, or influenza and are plotted against their respective Ad5 antibody titers. The bottom two rows are the percentages of CFSE low CD4 T cells and $\alpha_4\beta_7$ fold increases in response to the same antigens. *R* and *P* values were obtained using the Spearman test. Continued and dotted lines represent the best fit line and 95% confidence intervals respectively.

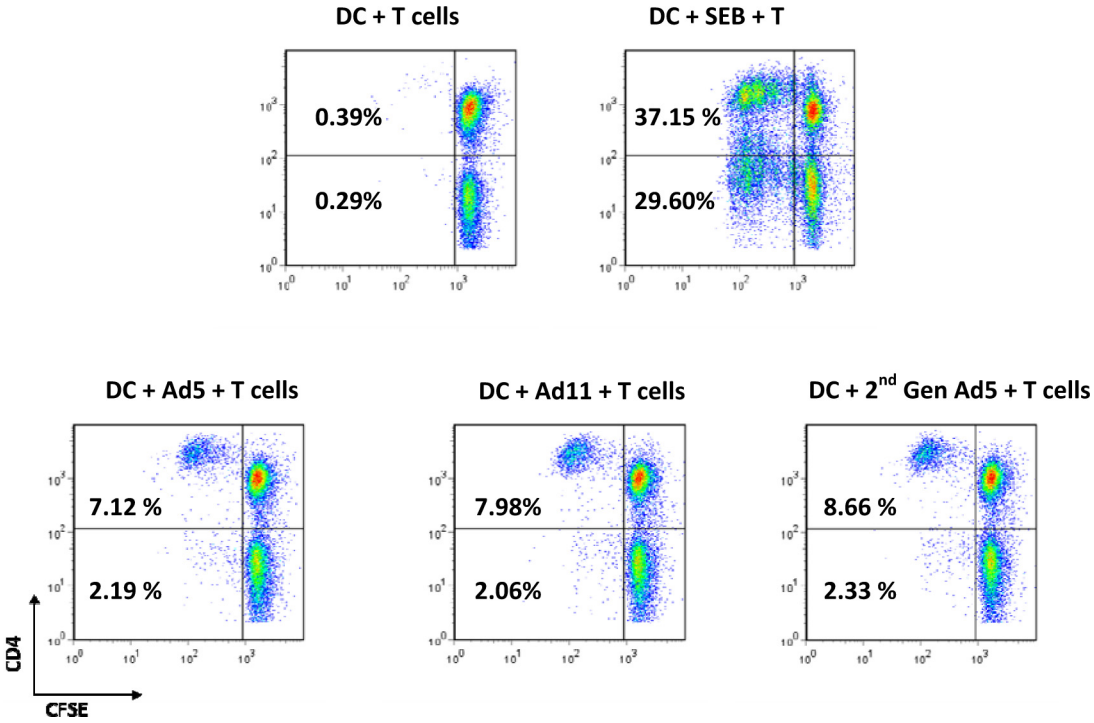

Fig. S3. Adenovirus-specific memory CD4 T-cell proliferation. CFSE-labeled lymphocytes were co-cultured with unstimulated or Ad5, Ad11, second-generation Ad5, or SEB-pulsed autologous DCs. Dot plots from a representative sample show the proliferation of the live $CD3^+$ population.

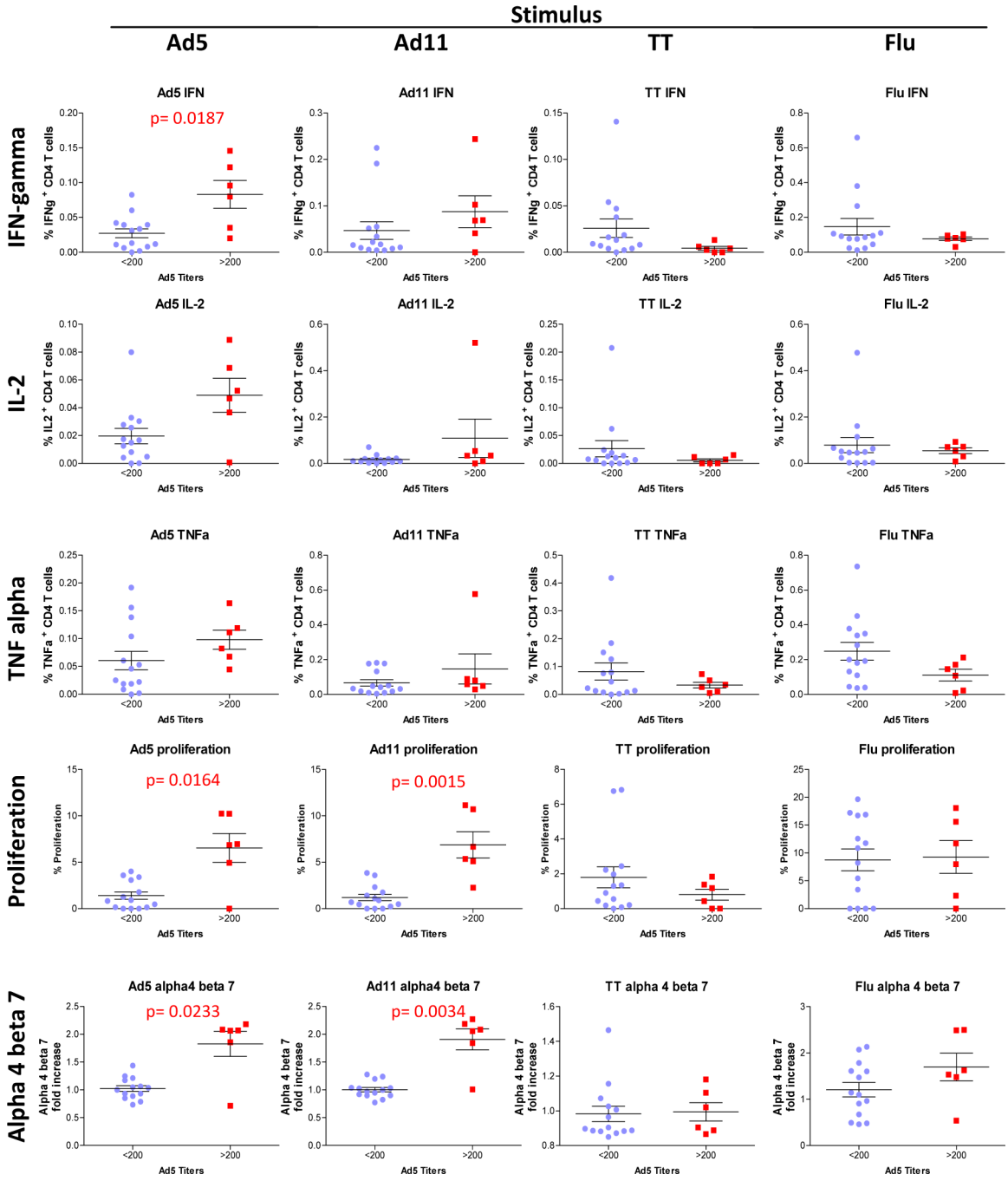

Fig. S4. The first three rows of plots represent the percentages of IFN-γ, IL-2, and TNF-α producing CD4 T-cell in response to (from left to right) Ad5, Ad11, tetanus toxoid, or influenza and are shown for individuals with Ad5 titers less than or equal to 200 (blue circles) and those with Ad5 titers >200 (red squares). The bottom two rows are the percentages of CFSE-low CD4 T cells and $\alpha_4\beta_7$ fold increases in response to the same antigens. *P* values <0.05 are given (Mann Whitney U test).

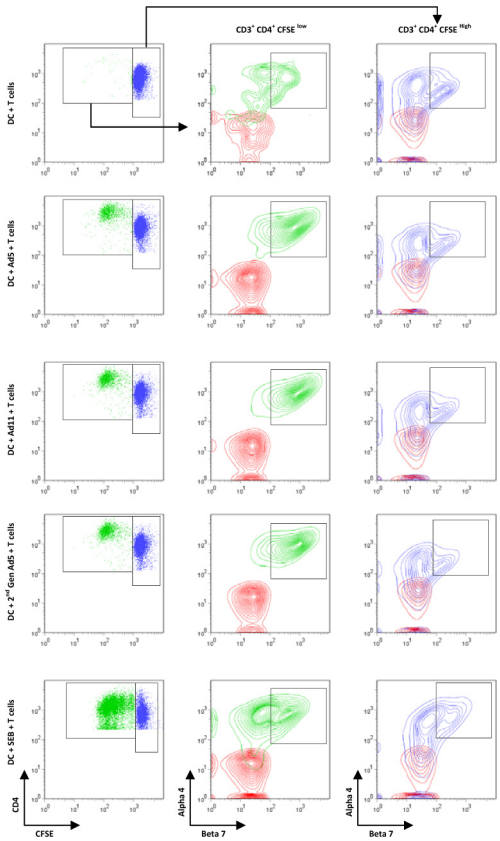

Fig. S5. $\alpha_4\beta_7$ expression by adenovirus-specific memory CD4 T cells. From top to bottom, lymphocytes were co-cultured in the presence of unstimulated, Ad5, Ad11, second-generation Ad5, or SEB-pulsed autologous DCs. Dot plots from a representative sample (left column) show CFSE staining of CD3+ $CD4^+$ T lymphocytes after 4 days of proliferation. $\alpha_4\beta_7$ expression by CFSE low (green contours) or CFSE high (blue contours) are shown in the middle and right columns, respectively, while red contours represent isotype controls.

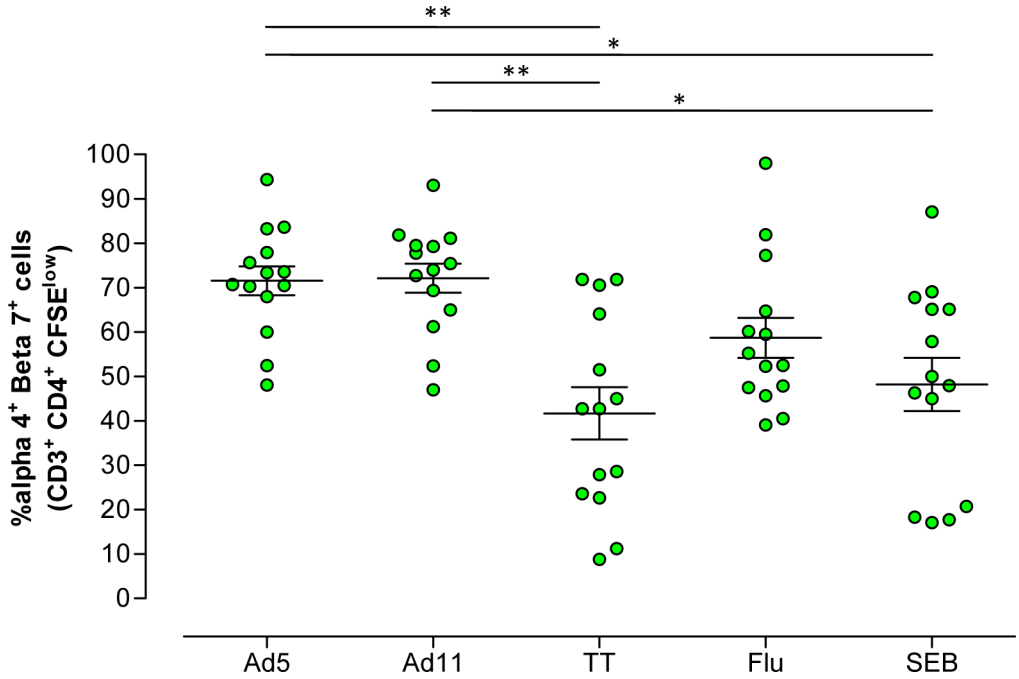

Fig. S6. $\alpha_4\beta_7$ expression by memory T cells. Fourteen samples that showed a proliferative response against Ad5 and Ad11 were also stimulated with tetanus toxoid, influenza, and SEB. $\alpha_4\beta_7$ expression by expanded CFSE low $CD3^+$ $CD4^+$ T cells is shown. Bars, means ± SD.) *, **, and *** indicate *P* values of less than 0.05, 0.01, and 0.001, respectively (Mann Whitney U test).

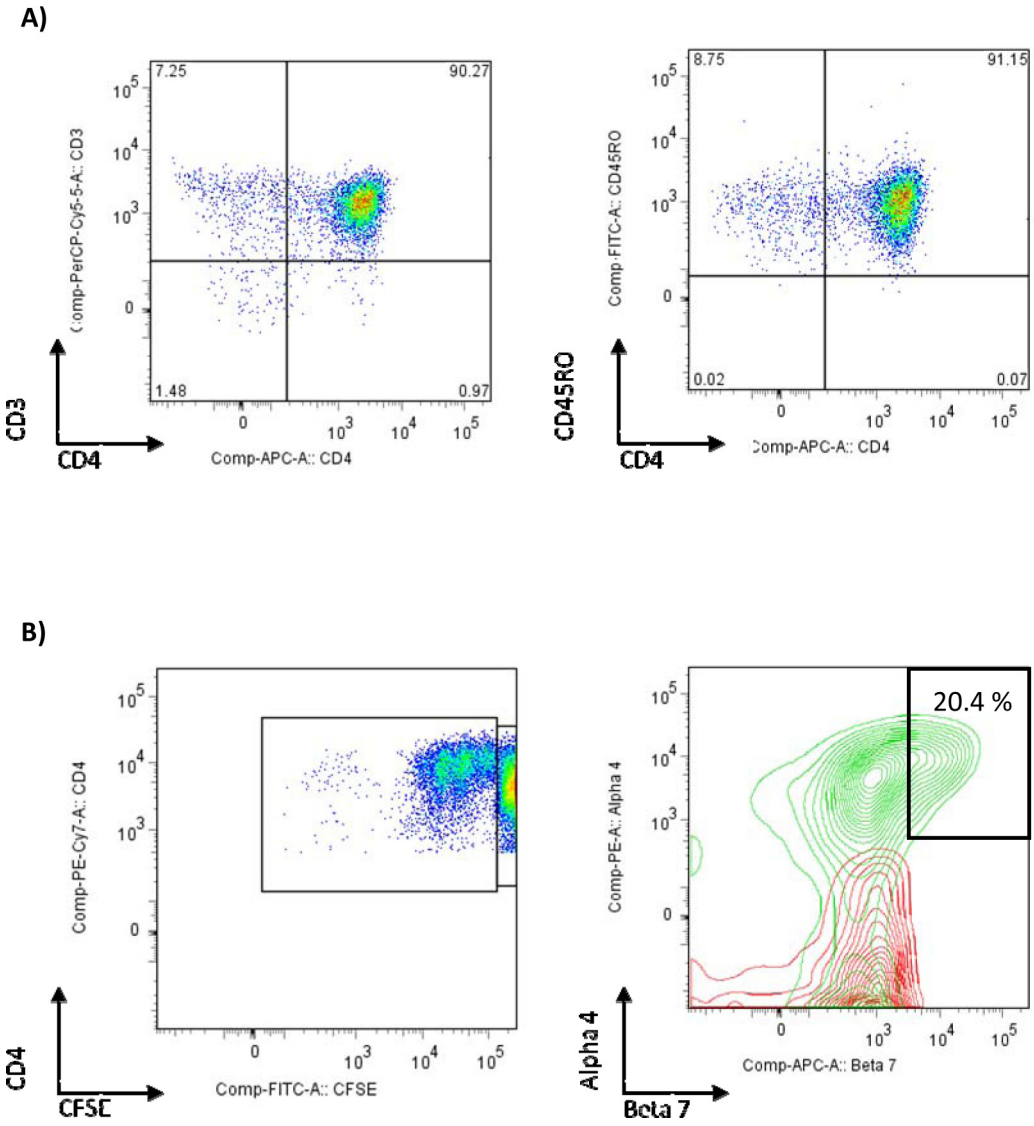

Fig. S7. (*A*) phenotype of purified memory CD4 T cells ($n = 2$). (*B*) Isolated memory CD4 T cells were co-cultured with autologous SEB stimulated DC for 4 days. $\alpha_4\beta_7$ expression by the proliferating cells (CFSE low, green) is shown. Red contours represent the isotype controls.

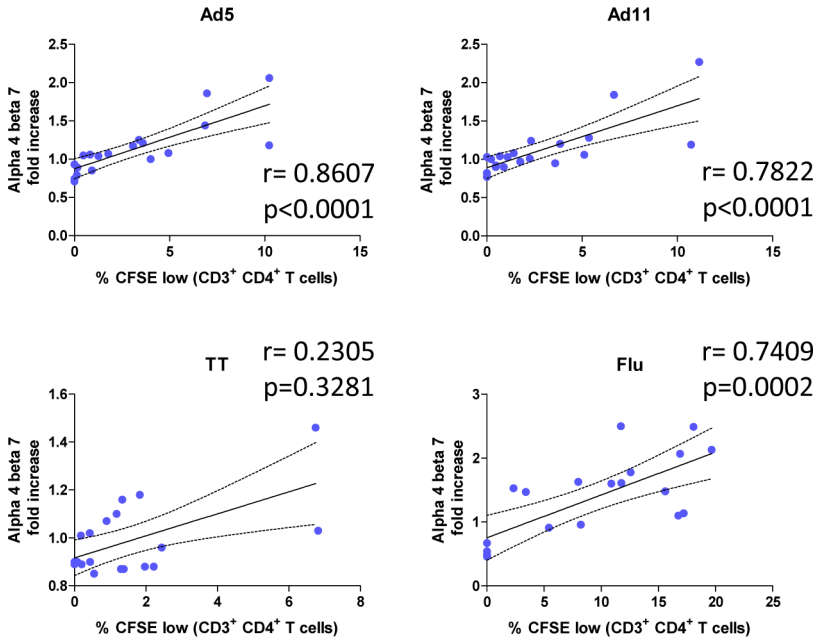

Fig. S8. $\alpha_4\beta_7$ increases were plotted against the percentages of proliferating CFSE-low CD4 T cells in response to Ad5 (top left), Ad11 (top right), tetanus toxoid (bottom left), or heat-inactivated influenza (bottom right). *R* and *P* values were obtained using the nonparametric Spearman test. Continued and dotted lines represent the best fit line and 95% confidence intervals respectively.

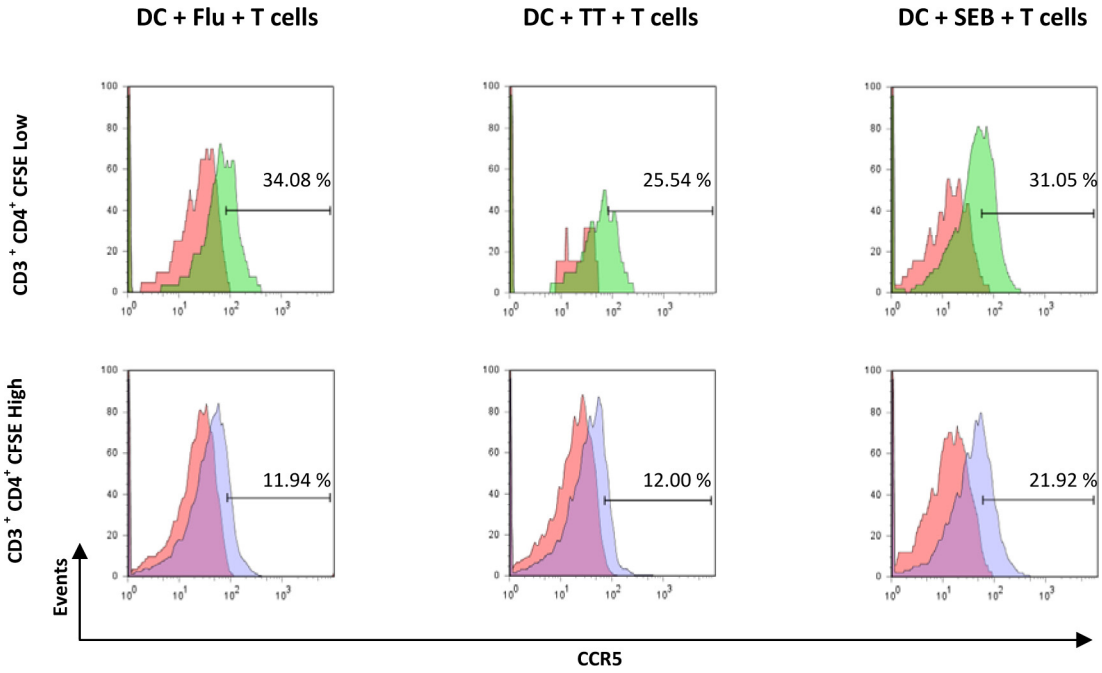

Fig. S9. CCR5 expression by expanded memory CD4 T cells. CFSE-labeled T cells were co-cultured with Flu, tetanus toxoid, or SEB-pulsed DC. CCR5 expression by divided CFSE-low (green histograms) or undivided CFSE-high $CD3^+$ $CD4^+$ T lymphocytes (blue histograms) was measured by flow cytometry. Red histograms represent the non-specific staining using the appropriate isotype controls. Plots are representative of 4–8 samples.

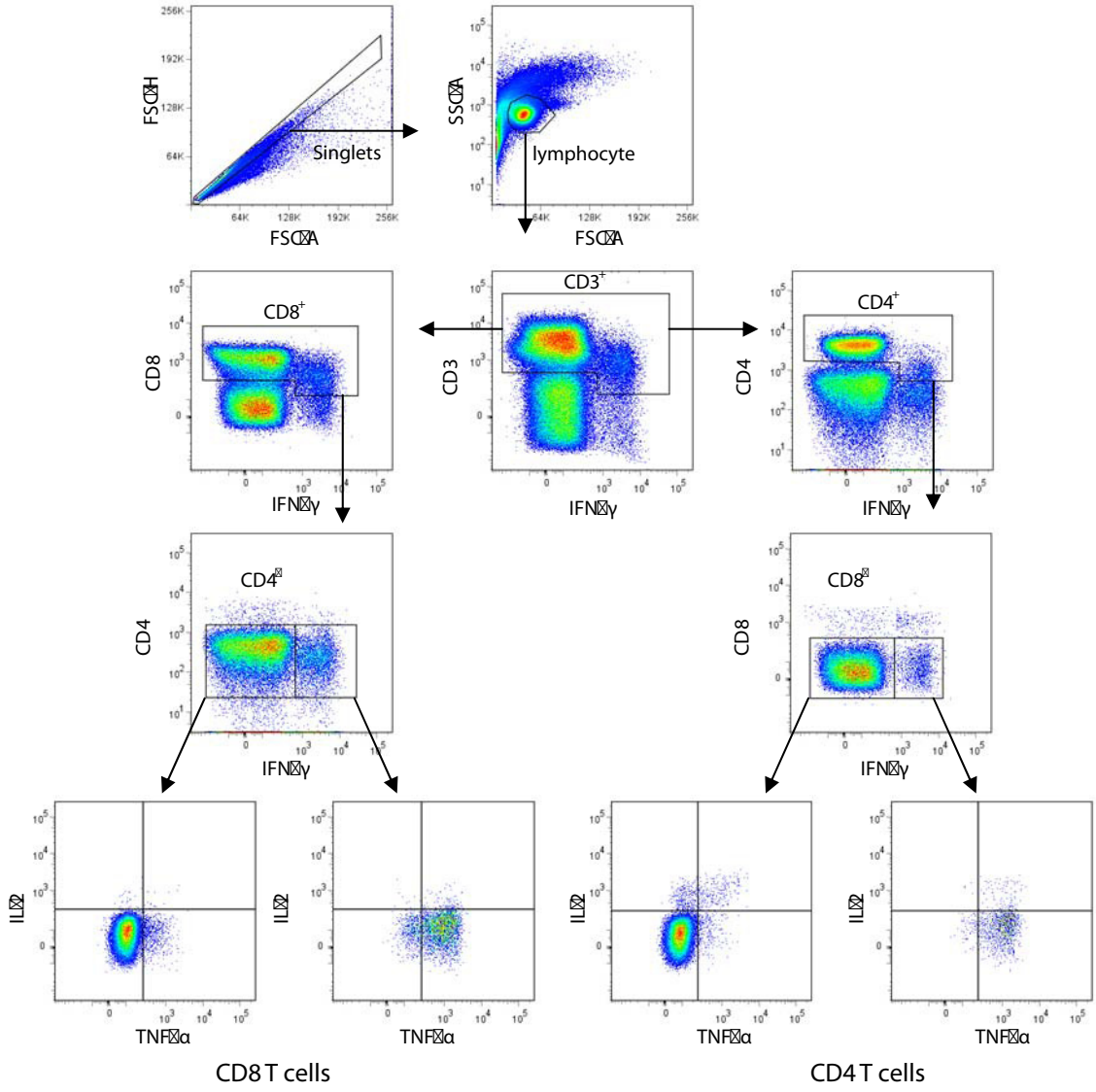

Fig. S10. Gating strategy for the detection of multifunctional CD4 and CD8 T-cell responses.