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Title: Baseline Ad5 serostatus does not predict Ad5-HIV vaccine-induced expansion of Ad-specific CD4+ T-cells

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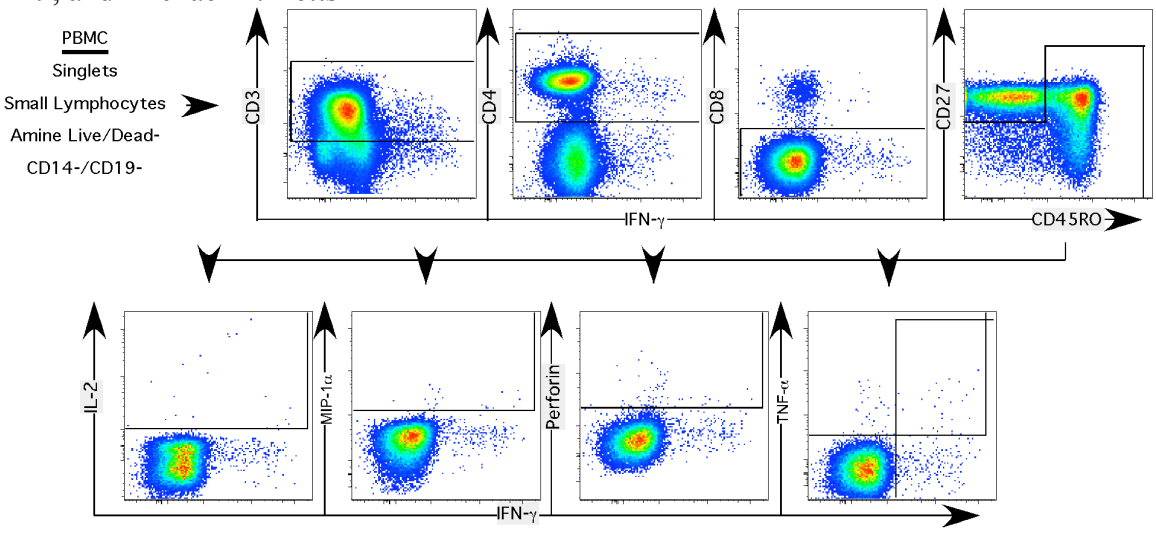
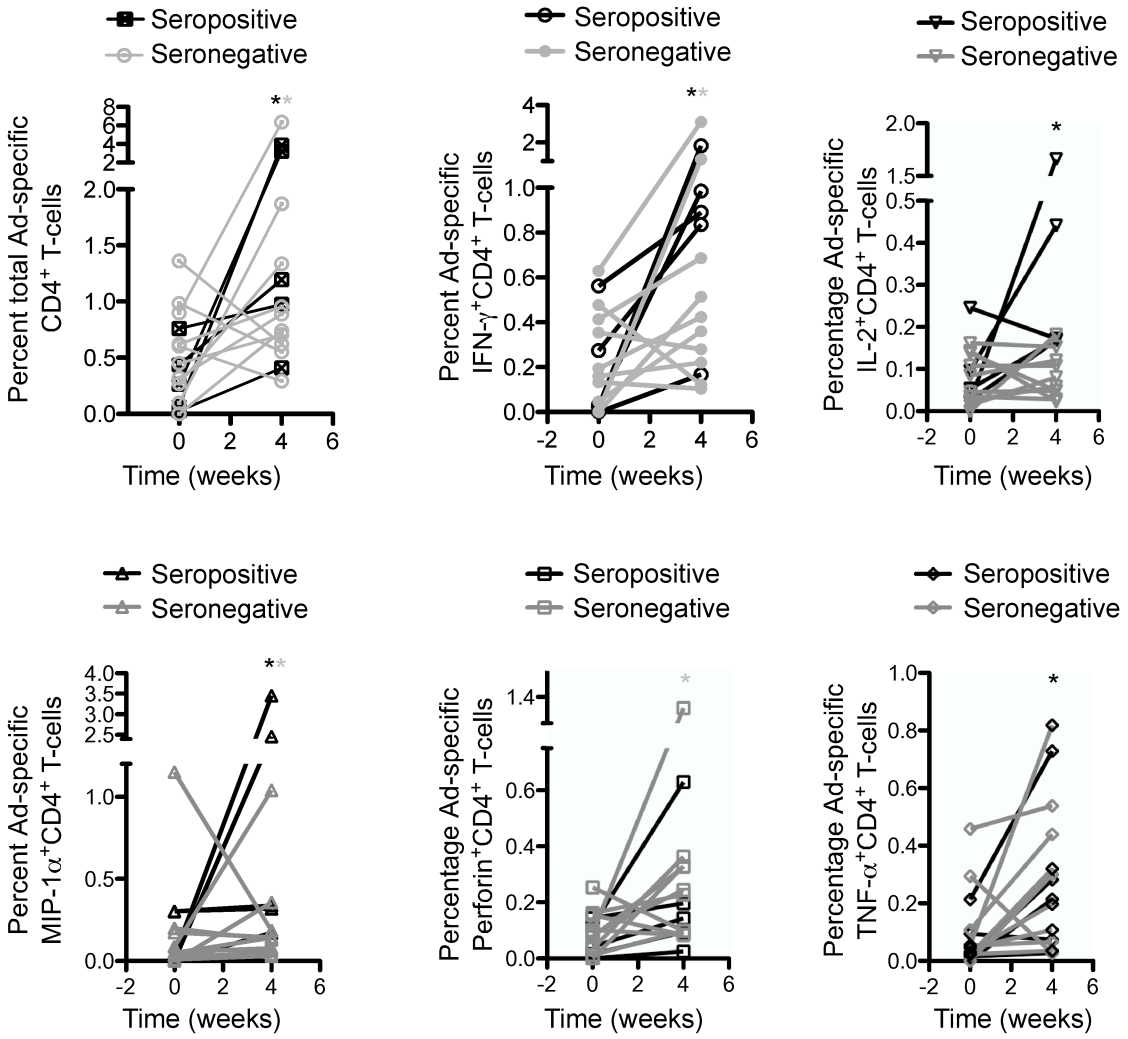
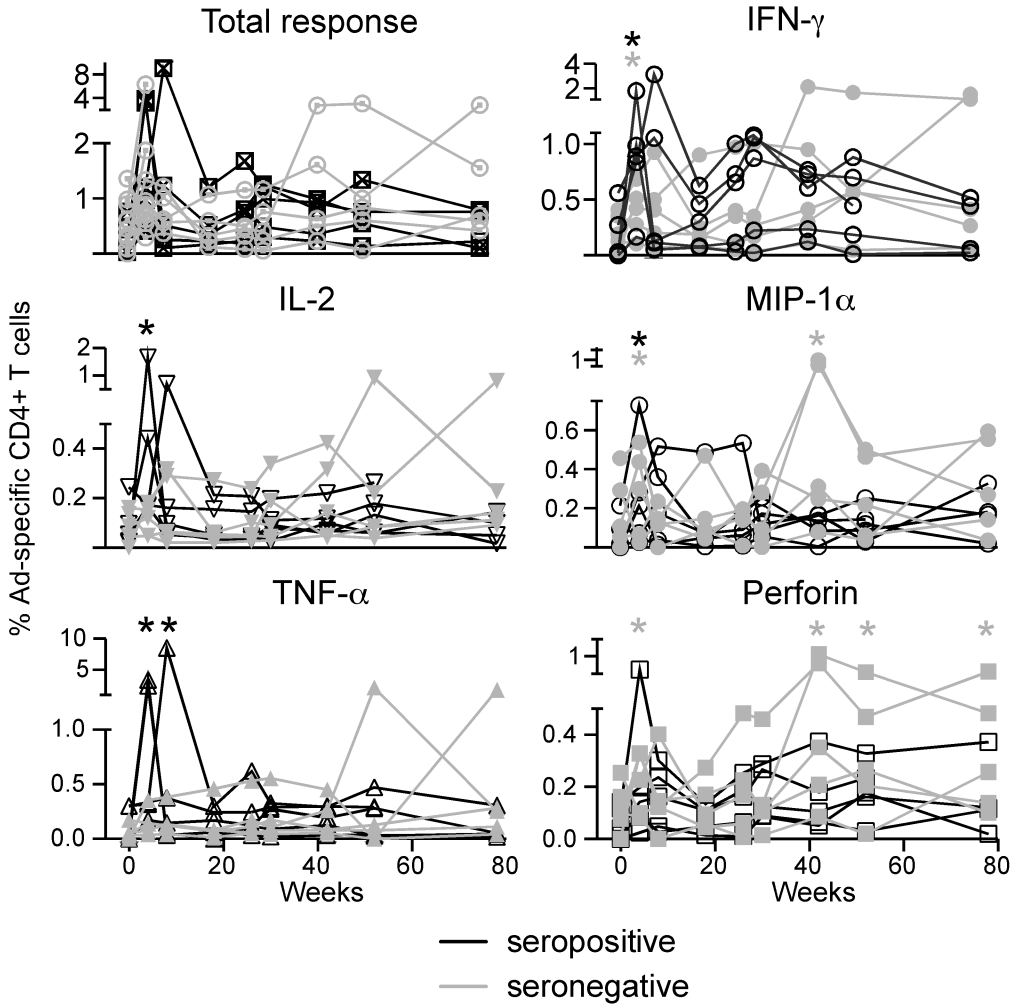


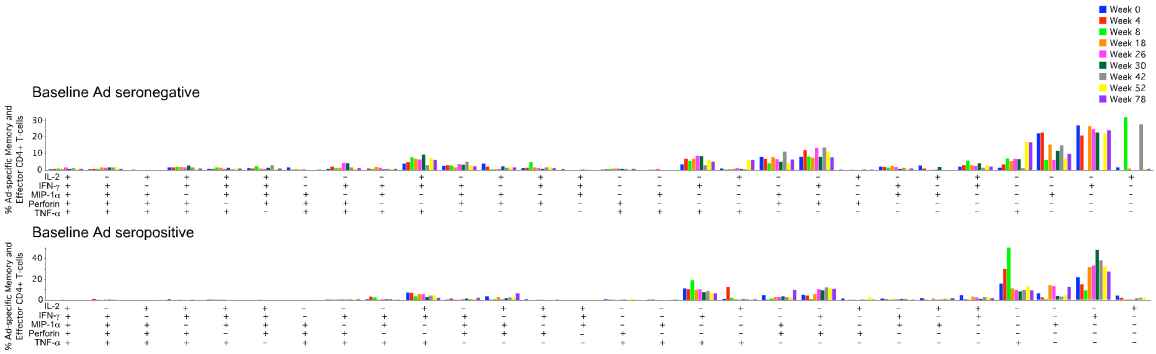
Supplementary Figure 1: Gating strategy for measuring Ad5-specific T-cells by intracellular cytokine staining. We collected at least 100,000 PBMCs with a threshold of 25,000. Singlets were selected with a FSC-A and FSC-H, followed by a lymphocytes gate, dead cell exclusion, and exclusion of contaminating $CD14^+$ monocytes and $CD19^+$ B-cells. We selected T-cell by $CD3^+$ and then $CD4^+$ cells by $CD4^+CD8^-$. Central memory, effector memory and effector $CD4^+$ T-cells were selected before gating on each cytokine. Because cells can store perforin and these appear $perforin^+$, Ad5-specific $CD4^+perforin^+$ T-cells must also be positive for another function to be considered as a responding event.



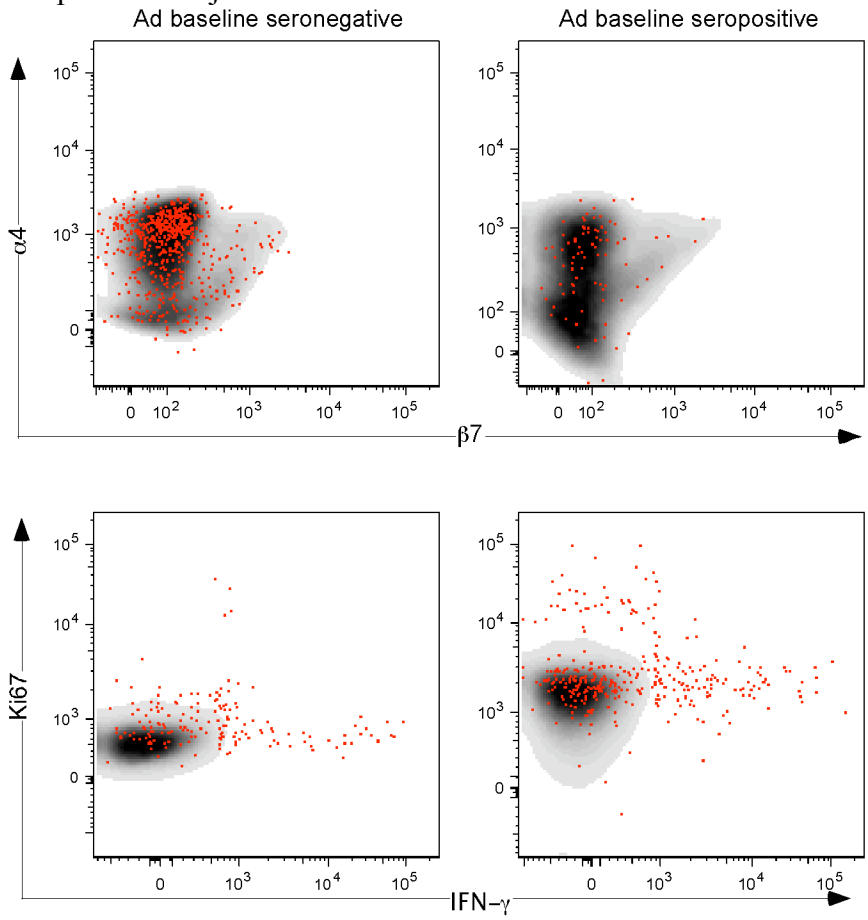
Supplementary Figure 2: Ad5-specific T-cell responses following a single vaccination. Seronegative subjects (n = ten, grey) and seropositive subjects (n = five, black) received $3x10^{10}$ vp Merck Ad5 gag/pol/nef at week 0. We measured the percentage of cytokine^{+} CD4^{+} T-cells by intracellular flow cytometry and all results are background subtracted. In both groups, total responses were elevated at week 4 compared to baseline ($P < 0.002$, baseline seropositive; $P < 0.03$, baseline seronegative). In seropositive subjects IFN-γ ($P < 0.002$), IL-2 ($P < 0.002$), MIP-1α ($P < 0.03$), and TNF-α ($P < 0.0001$) were significantly increased at week 4, while IFN-γ ($P < 0.03$), MIP-1α ($P < 0.01$), and perforin ($P < 0.001$) were significantly increased above baseline in seronegative subjects at week 4.



Supplemental Figure 3: Change in the percentage of Ad5-specific CD4$^+$cytokine$^+$ T-cells. Ten seronegative (Ad5 nAb titer ≤ 18, five weeks 0-4 and five weeks 0-78, grey) and five seropositive subjects (Ad5 nAb titer > 18, weeks 0-78, black) received 3x10^{10} vp Merck Ad5 gag/pol/nef at weeks 0, 4 and 26 with PBMCs collected at weeks 0, 4, 8, 18, 26, 30, 42, 52 and 78. We measured the percentage of CD4$^+$ IFN-γ, IL-2, MIP-1α , TNF-α, and perforin positive T-cells by intracellular flow cytometry. Ad5-specific CD4$^+$ IFN-γ$^+$ T-cell frequency increases after vaccination in Ad5 seropositives (*) at weeks 4 (P < 0.005), 8 (P < 0.05) and 30 (P < 0.5) and Ad5 seronegatives (*) at week 4 (P < 0.03). In seropositive subjects IL-2 increased above baseline (*) at week 4 (P < 0.03), MIP-1α at week 4 (P < 0.03), and TNF-α at weeks 4 (P < 0.0001) and 8 (P < 0.005); in seronegative subjects MIP-1α increased (*) above baseline at weeks 4 (P < 0.005) and 42 (P < 0.001), and perforin at weeks 4 (P < 0.001), 42 (P < 0.0001), 52 (P < 0.05) and 78 (P < 0.05).



Supplemental Figure 4: Polyfunctionality of Ad5-specific CD4$^+$ responses in vaccinated subjects. We stimulated PBMCs with whole Ad5 vector and cytokine responses were measured by intracellular cytokine staining. The percentage of cells making each possible combination of cytokines was calculated using the Boolean function in FlowJo. Bars represent the average percentage of responding Ad5-specific CD4$^+$ T-cells making each cytokine combination at each time point. Seronegative subjects demonstrated similar polyfunctionality at all time points compared with seropositive subjects.



Supplemental Figure 5: Representative flow plots of ki67 and a4/b7 staining. Data shown have been gated on CD3$^+$ CD4$^+$ T-cells as shown in **Supplemental Figure 1**. Grey density plots represent total memory CD4$^+$ T-cells. Red dot overlays represent Ad-specific CD4$^+$ T-cells as defined by production of one or more cytokine.

Supplemental Table 1

Marker	Function	Color
CD3	T-cell lineage	Qdot 585
CD4	T-cell lineage	PeCy5.5
CD8	T-cell lineage	Texas Red PE
live/dead	dead exclusion	Aqua
CD14	Monocytes	APCAl750
CD19	B-cells	APCAl750
CD27	Memory	PeCy5
CD45RO	Memory	Qdot 705
CD57	Memory	Qdot 565
MIP-1α	Chemokine	PE
Perforin	Lytic	Pacific Blue
TNF-α	Cytokine	PeCy7
IL-2	Cytokine	APC
IFN-γ	Cytokine	Alexa700

Supplemental Table 1: Flow cytometry antibody panel for intracellular cytokine staining. Qdot conjugates were prepared and tested in the Betts laboratory. All antibodies were titrated for optimum separation of positive and negative events when used as part of the panel

Supplemental Table 2

Group	N	Vaccine Protocol	Ad5 Serostatus at Baseline
baseline	25	Week 0 baseline non-vaccinated samples	15->2000
sernegative	5	$3x10^{10}$ vp MRK Ad5 HIV-1 gag/pol/nef wks 0, followed weeks 0 and 4	undetectable
seronegative	5	$3x10^{10}$ vp MRK Ad5 HIV-1 gag/pol/nef wks 0, 4, 26	undetectable
seropositive	5	$3x10^{10}$ vp MRK Ad5 HIV-1 gag/pol/nef wks 0, 4, 26	> 200

Supplemental Table 2: Study subjects. Baseline samples were available from 25 unvaccinated subjects with a range of Adenovirus 5 neutralizing antibody titers (Ad5 nAb) ranging from 18 (undetectable) to 2519. Five seronegative subjects received a single $3x10^{10}$ vp Merck Ad5 gag/pol/nef vaccine at week 0 and PBMCs were collected at weeks 0 and 4. Five seronegative (nAb titer ≤ 18) and five seropositive (nAb titer > 18) received three doses of $3x10^{10}$ vp at week 0, 4 and 26 and PBMCs were collected at weeks 0,4,8, 18, 26, 30, 42, 52 and 78. All subjects were HIV negative throughout the study.

Supplemental Methods

Subjects: We obtained frozen peripheral blood mononuclear cells (PBMCs) from unvaccinated subjects at baseline (*n* = 25), seronegative subjects (Ad5 neutralizing antibody titer ≤ 18) receiving $3x10^{10}$ vector particles (vp) MRKAd5HIV-1 gag/pol/nef at week 0, (*n* = five, followed week 0 and 4 only) seronegative subjects receiving three doses at weeks 0, 4 and 26 (*n* = five) and seropositive (Ad5 neutralizing antibody titer > 18) subjects receiving three doses at weeks 0, 4 and 26 (*n* = five) as part of a Merck phase I trial. Written informed consent was obtained from participants. The vaccination dose and schedule was identical to that used in the phase II STEP trial. PBMCs were obtained from study weeks 0, 4, 8, 18, 26, 30, 42, 52 and 78.

Vector: We prepared Adenovirus 5 (Ad5) vector using previously described methods[4]. The Ad5 vector was grown on HEK293 cells in DMEM supplemented with 10% fetal calf serum, antibiotics and glutamine[7]. Vector was purified by CsCl gradients and quality controlled.

Antibodies: We obtained directly conjugated antibodies from the following: BD Biosciences: TNF-α (Pe-Cy7), IFN-γ (Alexa700); Caltag: CD14 (APC-Alexa750), CD19 (APC-Alexa750), B7 Integrin (PeCy5), CD49d α4 (APC) Ki67 (Fitc), CD103 (FITC) and CD4 (Pe-Cy5.5); Beckman Coulter: CD8 (ECD), CD27 (Pe-Cy5); eBioscience CCR7 (APC-Alexa750) and R&D systems: MIP-1α (FITC), and IL-2 (APC). We conjugated the following antibodies in our laboratory: CD3 (QD585), CD57 (QD565), CD45RO (QD705), and Perforin (PacificBlue). We obtained the unconjugated CD45 and CD57 from AbD Serotec, perforin from Diaclone and CD3 OKT3 from American Type Culture Collection. We obtained Pacific Blue and Quantum Dots from Invitrogen.

Cell Stimulation and Staining: We measured T cell responses to E1-deleted Ad5 vector that expressed the rabies virus glycoprotein[5,6]. $2x10^6$ PBMCs were incubated overnight with $1x10^{11}$ Ad5 vp and costimulatory antibodies (αCD28 and 49d, 1 μg ml^{-1}; aCD28 alone for mucosal marker staining, BD Biosciences) at 37 °C and 5% CO_2 in 1 ml complete RPMI media (RMPI 1640 with 10% heat inactivated FBS, 100 U mL^{-1} Penicillin, 100 μg mL^{-1} streptomycin sulfate and 1.7 mM sodium glutamate). We stimulated a positive control was stimulated with *Staphylococcus* enterotoxin B (SEB, 1 mg ml^{-1}; Sigma-Aldrich) and a negative control received only co-stimulatory antibodies. The following morning we added Monensin (Golgi Stop, 0.7 μg ml^{-1}; BD Biosciences) and Brefeldin A (1 μg ml^{-1}; Sigma-Aldrich) to each sample and incubated the cells for six hr at 37 °C and 5% CO_2. We then washed samples in PBS and stained for viability (Aqua live/dead amine reactive dye; Invitrogen) followed by treatment with surface antibodies. We washed the cells, permeabilized and fixed using the Cytofix/Cytoperm kit (BD Biosciences) then stained with intracellular fluorochome-labeled antibodies. Following staining cells were washed, fixed (2% paraformaldehyde in PBS) and stored at 4 °C in the dark until analysis.

Flow Cytometry: We analyzed cells on a modified LSR II flow cytometer (BD Immunocytometry Systems) with 200,000 to 1,000,000 events collected per sample. We analyzed data using FlowJo 8.7.1 (TreeStar). Cells were initially gated to remove

doublets followed by a lymphocytes gate and forward scatter area versus side scatter area. We removed dead cells by gating CD3 versus Aqua blue removing dead cells that are Aqua blue bright. We removed contaminating $CD14^+$ and $CD19^+$ cells before gating sequentially on $CD3^+$, $CD8^+/CD4^+$ and $CD4^-/CD8^-$ versus IFN-γ to account for receptor down-regulation. We then made a gate for each respective function and the Boolean gating platform used to create the array of 32 possible functional combinations. Data are reported after background subtraction of the no stimulation condition.

nAb titers: We measured Adenovirus 5 neutralizing antibody titers as previously described[9]. Briefly, $2x10^4$ HEK293 cells per well in a 96 well plate were seeded for 2 days. Ad-SEAP was incubated for 1 hour at 37 °C either alone or with serial dilutions of serum then added to the 95-100% confluent 293 cells and incubated for 1 hr at 37 °C. Supernatant was then removed and replaced with 10% FBS in DMEM. DEAP expression as measured 24 ± 2 hrs later with the chemiluminescent substrate from the Phospha-Light™ kit. (Applied Biosystems).

Statistics: we performed mixed effects models to test for group differences over time. we also used mixed effects models for comparisons between baseline and subsequent time points within each group. Time was considered to be a discrete variable, lessening the power of these tests compared to tests where time is a continuous variable. We used Spearman correlations to test the relationship between Ad5 nAb titers and T cell functions at baseline. Correlations over the entire time period were computed using partial correlation coefficients controlling for individual subject effects in the repeated measurements. We log-transformed all data using base e.