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Baseline Ad5 serostatus does not predict Ad5 HIV vaccine–induced expansion of adenovirus-specific CD4$^+$ T cells

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The mechanisms underlying possible increased HIV-1 acquisition in adenovirus 5 (Ad5)-seropositive subjects vaccinated with Ad5–HIV-1 vectors in the Merck STEP trial remain unclear. We find that Ad5 serostatus does not predict Ad5-specific CD4$^+$ T cell frequency, and we did not observe durable significant differences in Ad5-specific CD4$^+$ T cells between Ad5-seropositive and Ad5-seronegative subjects after vaccination. These findings indicate no causative role for Ad5-specific CD4$^+$ T cells in increasing HIV-1 susceptibility in the STEP trial.

Post hoc analysis of the Merck STEP trial showed that vaccination with an Ad5 vector–based HIV-1 vaccine was associated with increased HIV-1 acquisition rates in volunteers with baseline Ad5-neutralizing antibody titers of >200 (ref. 1). It was proposed that vaccination of Ad5-seropositive subjects caused activation and expansion of preexisting Ad5-specific CD4$^+$ T cells potentially serving as targets for HIV-1 infection[2]. However, neither the prevalence of Ad5-specific CD4$^+$ T cells in humans nor their relationship with Ad5-neutralizing antibody titer has been characterized. Moreover, it is unknown to what degree Ad5 vector administration stimulates preexisting Ad5-specific CD4$^+$ T cells. Adequately addressing this from the STEP trial is impossible, as peripheral blood mononuclear cell samples were only obtained after vaccination (weeks 8, 30, 52 and 104)[3].

To characterize the relationship between Ad5-neutralizing antibody titers and Ad5-specific CD4$^+$ T cell responses, we analyzed samples from 40 subjects with varying Ad5-neutralizing antibody titers by intracellular cytokine staining using replication-defective Ad5 particles for stimulation[4–7]. (**Supplementary Methods, Supplementary Fig. 1** and **Supplementary Table 1**). Of these subjects, 15 (five baseline seronegative subjects followed weeks 0–4, five baseline seronegative subjects followed weeks 0–78 and five baseline seropositive subjects followed weeks 0–78) were enrolled in the Merck 016 phase 1 HIV-1 vaccine safety trial and received the Ad5 vectors used in the STEP trial at weeks 0, 4 and 26 (ref. 8 and **Supplementary Table 2**). We detected similar frequencies of Ad5-specific CD4$^+$ T cells in >80% of Ad5-seropositive and Ad5-seronegative subjects at baseline (**Fig. 1a**). Within Ad5-seropositive subjects, Ad5-specific CD4$^+$ T cell frequencies did not correlate with Ad5-neutralizing antibody titers[9] (**Fig. 1b**).

Four weeks after the first Ad5 vector HIV-1 vaccine administration in the 15 vaccinated subjects (**Supplementary Table 2**), Ad5-neutralizing antibody titers in baseline Ad5-seronegative subjects ($n = 10$) increased ($P < 0.05$), becoming comparable to those seen in baseline Ad5-seropositive subjects ($n = 5$) in all but one individual (**Fig. 1c**) who seroconverted by week 8 (**Fig. 1d**). Ad5-specific CD4$^+$ T cell numbers increased in both groups ($P < 0.002$, baseline seropositive; $P < 0.03$, baseline seronegative) after the initial vector dose (**Fig. 1e** and **Supplementary Fig. 2**). Successive vaccinations further expanded Ad5-specific T cells in some subjects, but these responses were transient in most individuals (**Fig. 1e** and **Supplementary Fig. 3**). At no point was there a statistical difference between the serogroups.

We next examined the relationship between Ad5 serostatus and potential functional differences in Ad5-specific CD4$^+$ T cells before and after vaccination. Ad5-specific CD4$^+$ T cells that produced interferon-γ (IFN-γ), interleukin-2 (IL-2), macrophage inflammatory protein-1α (MIP-1α), tumor necrosis factor-α (TNF-α) and/or perforin were present at baseline in most individuals at similar frequencies, regardless of Ad5 serostatus (**Fig. 2a**). There was no correlation between Ad5-neutralizing antibody titer and the percentage of Ad5-specific CD4$^+$ T cells that produced any one or more functions (data not shown). IFN-γ dominated the response in both serogroups but accounted for only ~50% of the total response (**Fig. 2b**).

After the first vaccination, Ad5-specific CD4$^+$IFN-γ$^+$ T cell counts increased in both serogroups ($P < 0.05$), with the fold change in CD4$^+$IFN-γ$^+$ T cell counts independent of serostatus (**Fig. 2c** and **Supplementary Figs. 2–4**). The frequency of Ad5-specific MIP-1α$^+$ CD4$^+$ T cells increased above baseline after vaccination ($P < 0.03$, seropositive individuals; $P < 0.005$, seronegative individuals), whereas Ad5-specific IL-2$^+$ ($P < 0.03$) and TNF-α$^+$ CD4$^+$ T cells ($P < 0.001$) increased in seropositive subjects only and accounted for a higher proportion of the total response ($P < 0.05$) compared with that in seronegative subjects (**Fig. 2c–d** and **Supplementary Figs. 2** and **3**). After three vector doses, the percentage of Ad-specific CD4$^+$ T cells expressing perforin and MIP-1α were higher in seronegative subjects above baseline, but we found few differences in other functions in either serogroup after the second vaccine dose (**Fig. 2d** and **Supplementary Fig. 3**). Despite these transient increases in CD4$^+$ T cell functions within serogroups, there was never a significant difference between the groups for the percentage of Ad5-specific CD4$^+$ T cells producing IFN-γ, IL-2, MIP-1α, TNF-α or perforin. The degree of polyfunctionality of Ad5-specific CD4$^+$ T cells remained comparable between baseline Ad5-seronegative and Ad5-seropositive subjects (**Supplementary Fig. 4**).

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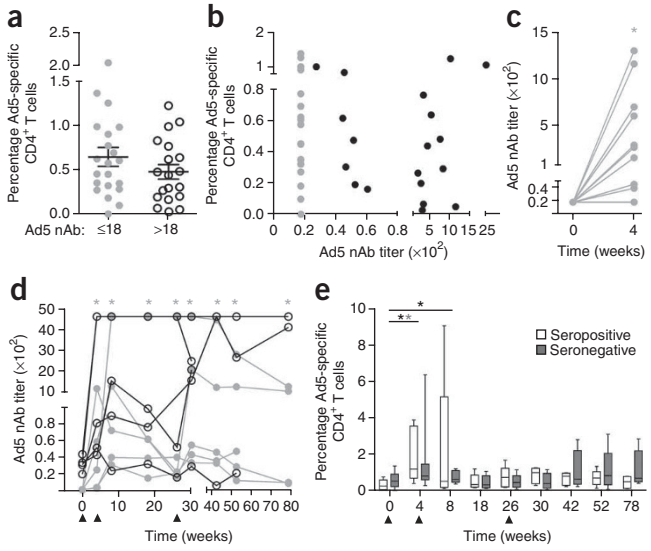

Figure 1 Flow cytometric analysis of Ad5-specific CD4+ T cell frequency. Forty subjects with a range of Ad5-neutralizing antibody (nAb) titers were analyzed. Ten seronegative (five assessed at weeks 0–4 and five assessed at weeks 0–78) and five seropositive subjects received Merck Ad5 emcoding HIV-1 gag, pol and nef as described in the **Supplementary Methods**. For all panels, gray symbols indicate baseline Ad5-seronegative subjects, and black symbols represent baseline Ad5-seropositive subjects. (**a**) Magnitude of baseline Ad5-specific CD4+ T-cell response. Total magnitude was assessed by summing IFN-γ, IL-2, MIP-1α, TNF-α and perforin production as measured by polychromatic flow cytometry in response to Ad5 virus particle stimulation. Error bars represent the mean ± s.e.m. (**b**) Baseline correlation between Ad5-specific CD4+ T cells and Ad5-neutralizing antibody (nAb) titer. (**c**) Ad5 neutralizing antibodies titers in Ad5 seronegative individuals after one vaccination. Ad5 nAb titer was significantly increased from baseline at week 4 (gray asterisk, $P < 0.05$). (**d**) Ad5-neutralizing antibody titers throughout the vaccine course. Ad5 nAb titers were significantly elevated in baseline seronegative subjects weeks 4-78 (gray asterisk, $P < 0.05$). Triangles indicate vaccination time points. (**e**) Ad5-specific CD4+ T cell frequency before and after vaccination. Ad5-specific CD4+ T cell frequency increases after vaccination in Ad5-seropositive individuals at weeks 4 (black asterisk, $P < 0.002$) and 8 ($P < 0.03$) and Ad5-seronegative individuals at week 4 (gray asterisk, $P < 0.02$). Plots depict the median, 25th and 75th percentiles (box plots) and the minimum and maximum values (whiskers). Triangles indicate vaccination time points.

We found no difference in expression of the proliferation marker Ki67 for total (data not shown) or Ad5-specific (**Fig. 2e** and **Supplementary Fig. 5**) CD4+ T cells between the serogroups or compared to baseline. Expression of the mucosal trafficking-associated markers α_4 and β_7 integrin did not differ substantially from baseline within either serogroup on total memory (T_M; all CD45RO+ and

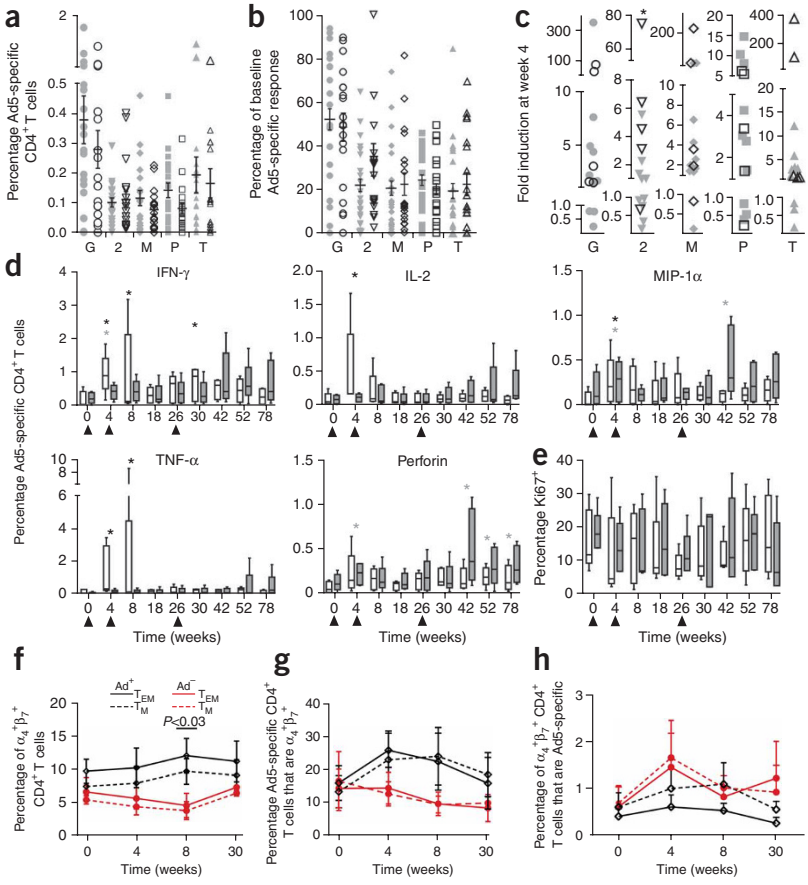

Figure 2 Flow cytometric analysis of CD4+ functionality, activation and mucosal homing. IL-2 (2), IFN-γ (G), MIP-1α (M), perforin (P) and TNF-α (T) production in response to Ad5 virus, as measured by intracellular cytokine staining. Subjects for all studies are as described in **Figure 1**. For all panels, gray symbols, lines or box plots depict baseline Ad5-seronegative subjects and open symbols, lines, or box plots depict baseline Ad5-seropositive subjects. (**a**) Percentage of baseline Ad5-specific CD4+ T cells producing various responses separated by Ad5 seropositivity. Error bars represent the mean ± s.e.m. (**b**) Percentage contribution of Ad5-specific CD4+ T cells expressing each indicated protein to the total Ad5-specific CD4+ T cell response at baseline. (**c**) Fold change in the indicated adenovirus-specific CD4+ T cell function after a single vaccination. IL-2 fold change was significantly higher in Ad5-seropositive individuals at week 4 (black asterisk, $P < 0.02$). (**d**) Changes in Ad-specific CD4+ T cell function after vaccination. In seropositive subjects, the percentage of Ad-specific CD4+IFN-γ+ T cells increased from baseline in the seropositive group at weeks 4 (black asterisk, $P < 0.005$), 8 ($P < 0.05$) and 30 ($P < 0.5$). The percentage of Ad-specific CD4+IL-2+ T cells was higher at week 4 ($P < 0.03$), the MIP-1α+CD4+ T cell percentage was higher at week 4 ($P < 0.03$) and TNF-α+CD4+ T cell percentage was higher at weeks 4 ($P < 0.0001$) and 8 ($P < 0.005$). In seronegative subjects, the IFN-γ+ CD4+ T cell percentage was higher (gray asterisk) than baseline at week 4 ($P < 0.03$), the MIP-1α+ CD4+ T cell percentage was higher at weeks 4 ($P < 0.005$) and 42 ($P < 0.001$), and the perforin+CD4+ T cell percentage was higher at weeks 4 ($P < 0.001$), 42 ($P < 0.0001$), 52 ($P < 0.05$) and 78 ($P < 0.05$). Plots depict the medians, 25th and 75th percentiles (boxes) and the minimum and maximum values (whiskers). Triangles indicate vaccination time points. (**e**) Ki67 expression on adenovirus-specific CD4+ T cells does not change after vaccination. Plots depict the medians, 25th and 75th percentiles (boxes) and the minimum and maximum values (whiskers). Triangles indicate vaccination time points. (**f**) α_4 and β_7 integrin co-expression on T_M or T_{EM} CD4+ T cells. (**g**) Percentage of Ad5-specific CD4+ T_M or T_{EM} cells that express both α_4 and β_7 integrins. *P* value denotes a significant difference between the groups at week 8. (**h**) Percentage of $\alpha_4^+\beta_7^+$ T_M or T_{EM} CD4+ T cells that are Ad5 specific.

CCR7$^-$CD45RO$^-$) and effector memory (T_{EM}; CCR7$^-$CD45RO$^+$) CD4$^+$ T cells (**Fig. 2f** and **Supplementary Fig. 5**) or Ad5-specific T_M or T_{EM} CD4$^+$ T cells (**Fig. 2g**) after vaccination. Moreover, Ad5-specific CD4$^+$ T_M and T_{EM} cells represented a small fraction of total circulating $\alpha_4^+\beta_7^+$CD4$^+$ T cells, and their abundance did not markedly change after vaccination (**Fig. 2h**). Thus, although we detected transient changes in the phenotype and magnitude of Ad5-specific CD4$^+$ T cell responses within groups after vaccination, we did not observe substantial differences between groups.

Our results indicate that Ad5-specific CD4$^+$ T cells were unlikely to have had a role in the possible increased susceptibility to HIV-1 infection observed in the STEP trial. Three findings support this conclusion. First, baseline Ad5-neutralizing antibody titers are not predictive of Ad5-specific CD4$^+$ T cell responses. Second, Ad5-specific CD4$^+$ T cells within both baseline Ad5-seronegative and Ad5-seropositive subjects expand in response to adenoviral vector administration. Although we observed slightly higher responses early in baseline Ad5-seropositive individuals, these differences were extremely transient and would not account for the increased acquisition primarily observed at later times (after week 20) in the STEP study. Thus, vaccination does not seem to preferentially increase the pool of potentially infectable circulating Ad5-specific CD4$^+$ T cells in Ad5-seropositive individuals. Last, Ad5-neutralizing antibody seronegative subjects uniformly became Ad5 seropositive after a single vaccination, yet enhanced susceptibility in baseline Ad5-seronegative STEP participants was not observed after the first vaccine administration. Taken together, these data suggest that any linkage between Ad5 serostatus and an Ad5-specific T cell–related increase in HIV-1 acquisition should have been observed only early after the first vaccine dose because afterward, the Ad5-specific T cell responses in baseline Ad5-seronegative subjects seem immunologically equivalent to those in baseline Ad5-seropositive subjects. With the caveat that our analyses are restricted to circulating CD4$^+$ T cells and do not address potential differences in activated Ad5-specific CD4$^+$ T cells within mucosal tissues after vaccination, our results do not support the hypothesis that Ad5-specific CD4$^+$ T cells contributed to the potential increased HIV-1 acquisition in the STEP trial.

This 016 clinical trial by Merck and Company, Inc. under a US Food and Drug Administration–approved Investigational New Drug document. Informed consent was given by each subject before enrollment in the Merck 016 trial. The study was reviewed and approved by the Instutional Review Board at each study site location.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

N.A.H., H.C.J.E. and M.R.B. designed experiments and prepared the manuscript and figures. H.C.J.E. provided Ad5 vector for assays. N.A.H. and D.G.C. performed cytokine experiments. S.A.D. and K.S.C. assisted with sample and data transfer. L.K. determined Ad5-neutralizing antibody titers. D.R.C. and M.N.R. provided all vaccinee samples and helped design experiments. S.J.R. performed all statistical analysis.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

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Erratum: A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis

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In the version of this article initially published, the structure of PR-957 in Figure 1a was incorrect. An -NH group was missing. The error has been corrected in the HTML and PDF versions of the article.

Corrigendum: Baseline Ad5 serostatus does not predict Ad5 HIV vaccine–induced expansion of adenovirus-specific CD4$^+$ T cells

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In the version of this article initially published, George Makedonas was omitted from the author list. The error has been corrected in the HTML and PDF versions of the article.