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The effect of adenovirus-specific antibodies on adenoviral vector–induced, transgene product–specific T cell responses

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ABSTRACT

In this study, we tested the effect of neutralizing Abs to different serotypes of E1-deleted Ad vectors on the immunogenicity of the homologous Ad vector or a vector derived from a heterologous serotype. Our results showed that, as expected, even low titers of passively transferred neutralizing Abs significantly reduced the homologous vectors' ability to elicit transgene-specific CD8$^+$ T cell responses. In addition, Abs changed the fate of transgene product–specific CD8$^+$ T cells by promoting their transition into the central memory cell pool, which resulted in markedly enhanced expansion of transgene product–specific CD8$^+$ T cells after a boost with a heterologous Ad vector. Non-neutralizing Abs specific to a distinct Ad serotype had no effect on the magnitude of transgene product-specific CD8$^+$ T cells induced by a heterologous Ad vector, nor did such Abs promote induction of more resting memory CD8$^+$ T cells. These results show that Abs to an Ad vaccine carrier affect not only the magnitude but also the profile of a vector-induced CD8$^+$ T cell response.
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Introduction

The phase IIB STEP trial tested the efficacy of an E1-deleted Ad vector of human serotype 5 (HAdV-5, also called AdHu5) in individuals at high risk for acquisition of HIV-1. Preclinically, the vector had shown protection of nonhuman primates challenged with the SIV–HIV chimera SHV89.6P [1], and early-phase trials had demonstrated the vaccine's immunogenicity in humans [2]. The vaccine, which was designed to induce cellular immunity of HIV-1 Ags, was tested in individuals who lacked VNAs to the vaccine carrier, as well as in those who were seropositive to the vector. The vaccine trial was stopped after an interim analysis showed lack of efficacy and increased HIV-1 acquisition among vaccine recipients who had pre-existing VNAs to AdHu5 [3, 4]. Furthermore, a human gene transfer study, conducted before the STEP trial, demonstrated that VNAs to the AdHu5 virus, which are common in humans [5], reduce the uptake of AdHu5 vectors and thus expression of the transgene product [6], impairing the vaccine's immunogenicity [7–9]. Notwithstanding, reduced immunogenicity in seropositive individuals does not explain the increased susceptibility to HIV-1 infection. The subsequent HVTN505 trial tested a regimen based on DNA vaccine priming, followed by an AdHu5 boost, with inserts designed to induce cellular and humoral immunity to Ags of HIV-1 in AdHu5-seronegative individuals. Again, the HVTN505 trial failed to show efficacy, and a nonsignificant trend toward higher infection rates was observed in vaccinated human subjects compared with placebo recipients. Although several theories were formulated and tested to explain the increased acquisition rates in the STEP trial [10, 11], the reasons for the apparent increases in susceptibility to HIV-1 infection in AdHu5 vaccine recipients remain elusive.

Vectors based on alternative human serotypes such as HAdV-26 (AdHu26) or HAdV-35 have been developed and tested [12, 13]. Depending on the geographic region, humans either lack VNAs to these serotypes or they carry markedly lower titers [14, 15]. Vectors derived from chimpanzee adenoviruses (SAdV, also termed AdC) are also being explored [14, 16], as prevalence rates of such viruses are even lower than those of alternative human serotypes [15]. The question of whether these alternative Ad vectors will outperform AdHu5-based HIV-1 vaccines in clinical trials or elicit the previously observed increased risk of HIV-1 infection in vaccine recipients remains open.

Ad=adenovirus; AdC=chimpanzee serotype adenovirus; AdHu=human serotype adenovirus; ELISA=enzyme-linked immunosorbent assay; gag=group-specific antigen (protein); GFP=green fluorescent protein; HAdV=vector based on a human adenovirus serotype; HIVgag=gag of HIV-1; KLRG1=killer cell lectin-like receptor subfamily G member 1; LSD=least significant difference; NPOVAGFP=influenza nucleoprotein fused to green fluorescent protein by the immunodominant class 1 restricted epitope of ovalbumin; PBMC=peripheral blood mononuclear cells; PBS-T=PBS Tween; qPCR=quantitative PCR; Rab.gp=rabies virus glycoprotein; RBC=red blood cell; SAdV=vector based on a simian adenovirus serotype; SIV=simian immunodeficiency virus; tet$^+$, tetramer positive; VNA=virus neutralizing Ab; vp=virus particles

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We tested whether VNAs to different serotypes of E1-deleted Ad vectors differentially affect the immunogenicity of the homologous vector. Although Ad-specific VNAs are serotype specific and are mainly directed to the hypervariable loops of the viral hexon, nonneutralizing Abs to more conserved regions of the viral surface proteins, such as the hexon stalk, cross-react between different human and simian serotypes [9]. We therefore also assessed the effect of passive transfer of Ad-specific Abs on the immune response induced by vaccination with an Ad vector derived from a heterologous serotype.

Our results confirmed that even low titers of passively transferred Abs significantly reduce the vectors' ability to elicit transgene product–specific $CD8^+$ T cell responses and that there was no definite difference between the distinct serotypes. Of interest, passive transfer of Ad VNAs changed the fate of transgene product–specific $CD8^+$ T cells by promoting their transition into the central memory cell pool, resulting in enhanced expansion of these cells after a boost with a heterologous Ad vector. Induction of Ad Abs to 1 serotype had no effect on the magnitude of transgene product–specific $CD8^+$ T cells induced by an Ad vector of a different serotype, nor did such Abs promote induction of more resting memory $CD8^+$ T cells. As expected, Ad vector–specific VNAs reduced the amount of vector-derived transcript copies that were recovered from cells at the injection sites or in the lymphatic tissues. Nevertheless, even in the presence of pre-existing immunity, the Ad vectors established a persistent infection, making it unlikely that lack of continued exposure to Ag allows for better formation of central memory T cell responses. Overall, these results show that Abs to an Ad vaccine carrier affect not only the magnitude but also the profile of an Ad vector-induced $CD8^+$ T cell response.

MATERIALS AND METHODS

Mice

Female 6- to 8-wk old BALB/c mice were purchased from the National Cancer Institute (Frederick, MD, USA) and were housed in the vivarium at The Wistar Institute (Philadelphia, PA, USA). All experiments were performed in accordance with approved animal protocols.

Viral vectors

E1-deleted adenoviral vectors expressing gag of HIV-1 clade B (HIVgag), rabies virus glycoprotein (rab.gp), or the nucleoprotein of influenza A/PR8 virus fused to GFP and the SIINFEKL epitope (NPOVAGFP) were generated and quality controlled as described elsewhere [17].

Generation of immune sera

Groups of 20 BALB/c mice were immunized i.m. with 10^{11} vp of AdHu5rab.gp, AdHu26rab.gp, or AdC6rab.gp, with an i.m. boost injection 6 wk later of 10^{11} vp of the identical vector used for priming. Blood was collected 4 wk after the boost to confirm titers of Ad-specific VNAs. Mice immunized with AdHu5rab.gp and AdHu26rab.gp were euthanized 5 wk after the boost, sera were collected, and VNA titers were determined to be 1:13,000 and 1:40,000, respectively. Those immunized with AdC6rab.gp received a second boost injection and were euthanized 6 wk after administration of the third dose. The serum VNA titer was determined to be 1:20,000.

Titration of Ad-specific VNAs

Sera were incubated at 56°C for 30 min. Serial, 2-fold dilutions (100 μL starting at 1:20) were incubated for 1 h at 37°C with a GFP-expressing Ad matching the serotype used for immunization. The virus–serum mixtures were added to HEK293 cells in 96-well plates and incubated overnight at 37°C. The cells were analyzed for GFP expression 24 h later. The VNA titer was determined as the highest dilution that resulted in <50% GFP-positive cells [5].

Administration of immune sera and immunization of mice

Immune sera were passively transferred into mice to achieve VNA titers of 1:1000, 1:100, and 1:10. The sera were diluted in sterile PBS to a volume of 200 μL per mouse and were administered intraperitoneally. The control group received naive BALB/c serum diluted to the highest dose of immune serum. Blood was again collected to confirm the appropriate VNA titers 5 days after transfer, and the mice were immunized i.m. the following day with 10^{10} vp of the vector diluted in 100 μL of sterile PBS.

ELISA

ELISA plates were coated with 10^8 vp/well of AdHu5-HIVgag, AdHu26-HIVgag, AdC6-HIVgag, or AdC7-HIVgag in 100 μL of coating buffer (2 carbonate-bicarbonate capsules [Sigma-Aldrich, St. Louis, MO, USA] dissolved in 100 mL H_2O) and incubated overnight at 4°C. The plates were washed with 0.05% PBS-T and blocked overnight at 4°C with 3% BSA in PBS-T. After the PBS-T wash, 2-fold serial dilutions of AdHu5, AdHu26, and AdC6 immune sera starting at 1:200 were incubated on the coated plates for 2 h at room temperature. The plates were washed with PBS-T, and AP-conjugated anti-mouse IgG diluted to 1:30,000 was applied to the plates for 1 h at room temperature. At the end of the incubation, the plates were washed, developed (phosphatase substrate dissolved in diethanolamine buffer), and read 30 min later at 405 nm. All samples were run in duplicate [9].

Lymphocyte isolation

Blood was collected via submandibular bleeding into 1 mL of 4% sodium citrate and 1 mL of L-15 (Cellgro, Manassas, VA, USA). PBMCs were isolated by gradient purification with underlying Histopaque-1083 (Sigma-Aldrich) and washed in PBS containing 1% FBS. The spleens were homogenized against a 70 μm filter screen and collected in L-15 containing 1% FBS. RBCs were lysed in RBC lysis buffer (eBioscience, San Diego, CA, USA). For isolation of lymphocytes from the genital tract, the vagina, cervix, uterine horns, and ovaries were removed and cut into fragments. Tissue segments were subjected to constant shaking at 130 rpm for 1 h in RPMI 1640 (Cellgro) containing 5% FBS and 1% penicillin-streptomycin (Sigma-Aldrich). The fragments were digested enzymatically with 1.4 mg/mL of collagenase type I (Life-Technologies, Grand Island, NY, USA) for 15 min. Cells from the 2 cycles were pooled and lymphocytes purified by a discontinuous Percoll gradient (Sigma-Aldrich) consisting of a 40% fraction containing cells overlaid on a 70% fraction [18].

Tetramer staining

Lymphocytes were stained with an APC-labeled tetramer against the immunodominant MHC class I restricted epitope of HIV-1gag (AMQMLKETI). In addition, the cells were stained with Abs to CD3, CD8, CD44, KLRG1 (Southern Biotech, Birmingham, AL, USA), CD62L (BD Pharmingen, San Jose, CA, USA), CD127, and CCR7 (eBioscience). Unless otherwise indicated, the Abs were from Biolegend (San Diego, CA, USA). A stain for dead cells was included in the panel [18].

Data acquisition and analysis

Samples were run on a BD-LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo software (Tree Star, Ashland, OR,

USA). After lymphoid cell acquisition, doublets and dead cells were removed and sample gated on $CD3^+CD8^+$ T cells. The remaining cells were plotted as $tetramer^+$ against CD44. Data are shown as tet^+CD8^+ T cells/10^6 live lymphoid cells. If addition, tet^+CD44^+ cells were phenotyped for the markers described above. A gate on the $CD44^{lo}CD8^+$ T cells served an internal, naive control for phenotyping. In the genital tract, the lymphocytes were additionally gated on $CD3^+CD8^-$ T cells to assess the $CD4^+$ T cell population.

PCR

qPCR. Genomic DNA of muscle cells (25 mg), splenocytes (5×10^6 mononuclear cells), or lymph node cells (5×10^6 mononuclear cells) from mice injected with sera and an AdHu5-HIVgag vector was isolated with the DNeasy tissue isolation kit (Qiagen). DNA samples were stored at −20°C until used. The qPCR reaction was subsequently performed on an aliquot of each individual sample (2 μL of a total of 50 μL) with Power SYBR Green Master Mix (Applied Biosystems-Life Technologies, Foster City, CA, USA) at a final volume of 25 μL. For amplification of gag, 100 nM of the following primers were used: forward, ACCACATCTACCCTGCAGGAACAG, and reverse, AGGGTCTCTGTCATCCAATTCTTC. GAPDH was amplified in parallel, to normalize the samples with the following primers at 200 nM: forward, TGCCCCCATGTTTGTGATGG, and reverse, AATGCCAAAGTTGTCATGGATGACC. Both reactions consisted of 42 cycles of denaturation at 95°C for 15 s followed by annealing/extension at 60°C for 30 s. All real-time PCR reactions were performed in quadruplicate on an ABI Prism 7500 Fast Sequence Detection System (Life Technologies) set to run at a standard ramp speed. Standard curves used to calculate the amount of gag DNA in each sample were determined by serially diluting a plasmid encoding HIV-1gag from 10^7 to 10^3 copies/μL. The expression data for HIV-1gag were normalized to GAPDH to control for variability in the samples.

Nested PCR. The first PCR was performed with the primers described above for 25 cycles, followed by use of a 1 μL aliquot of the first PCR on a second PCR with the same forward primer and the reverse primer AATGCCAAAGTTGTCATGGATGACC for 40 cycles. The first PCR was performed at 94°C for 45 s, 55°C at 45 s, and 72°C at 30 s. The nested PCR was performed at 94°C for 30 s, 56°C at 30 s, and 72 °C at 30 s. Negative (water) and positive (gag plasmid) controls were included in the first PCR and then also amplified in the nested PCR. The PCR product (100 bp) was visualized by gel electrophoresis on a 1.5% agarose gel.

Statistical analysis

The significance of differences between the 2 populations was calculated by *t* tests with the Holm-Šídák correction for type 1 errors in multiple tests. For nonnormally distributed data, the differences were analyzed by the Mann-Whitney test. Differences between multiple groups were tested by ANOVA with the Dunnett correction for type 1 errors, Tukey's multiple comparison tests, or uncorrected Fisher's LSD, as indicated in the figure legends. Correlations were conducted with the Spearman method with the Bonferroni correction for type 1 errors. Data were analyzed with Prism 6 software (GraphPad, San Diego, CA, USA).

RESULTS

Effect of transfer of Ad-specific immune sera on the magnitude of transgene product–specific $CD8^+$ T cell responses to a homologous Ad vector

To assess whether Ad vectors based on 3 distinct species—AdHAdV-5 (vectors termed AdHu5), a common human serotype of family C of Adenovirideae; HAdV-26 (vectors termed AdHu26), a human serotype of family D; and S-AdV-23 (vectors termed AdC6), a chimpanzee-derived virus of family E—are differentially affected by pre-existing VNAs, we generated serotype-specific immune sera of BALB/c mice by repeated immunizations with AdHu5, AdHu26, or AdC6 vectors expressing rabies virus glycoprotein. The sera were tested for neutralization of the homologous virus and then transferred into naive, syngeneic mice at 3 different doses, so that recipient mice had circulating VNA titers of approximately 1:1000, 1:100, or 1:10 on transfer. Control animals received the highest dose of serum harvested from naive syngeneic mice. Five days after transfer, blood was collected to confirm the Ab titers, and the mice were immunized with 10^{10} vp of the same HIV-1 gag-expressing vector backbone used for induction of the transferred sera. At 10 d and 3 and 8 wk later, blood was collected, and the PBMCs were tested for gag-specific $CD8^+$ T cell responses by staining with an MHC class I–specific tetramer to the immunodominant epitope of gag in $H\text{-}2^d$ mice (**Fig. 1A–C**). As vaccination affected the overall number of circulating $CD8^+$ T cells (not shown), tet^+ cells were normalized to 10^6 live cells. Even low titers of 1:10 of AdHu5-specific VNAs caused a significant reduction in gag-specific $CD8^+$ T cells tested 10 d or 8 wk after immunization. The decrease in responses became more pronounced in mice that had received higher doses of the immune serum. Gag-specific $CD8^+$ T cell responses to the AdHu26gag vector peaked at 3 wk in blood and then contracted markedly, compared with those induced by the AdHu5gag vector. In AdHu26gag-immunized mice, all 3 doses of transferred VNAs caused significant decreases in the number of circulating gag-specific $CD8^+$ T cells at all the time points analyzed. Gag-specific $CD8^+$ T cell responses to AdC6gag were consistently sustained over the observation period and were reduced in the presence of AdC6-specific VNAs, although, at the earlier time point, a significant reduction was observed only at VNA titers of 1:100 or higher.

To determine whether transfer of Ad-specific VNAs reduces gag-specific $CD8^+$ T cell responses in other compartments, mice were euthanized 8 wk after immunization, and lymphocytes from spleens and the genital tract were tested. In the spleens, gag-specific $CD8^+$ T cell responses to AdHu5 and AdC6 vectors were reduced in the mice that received any dose of VNAs (Fig. 1D–F). In contrast, the inhibition of responses to the AdHu26 vector, which induced significantly lower responses by wk 8 after vaccination compared to the other 2 vectors, was significant only in the mice with titers of 1:1000 AdHu26-specific VNAs at the time of immunization. For analysis of genital T cells, lymphocytes from 5 mice were pooled. In the control mice, responses were comparable after AdHu5gag and AdC6gag vaccination but markedly lower after AdHu26 immunization (Fig. 1G–I). The effects of VNAs on genital gag-specific $CD8^+$ T cells mirrored those observed in the spleens, but only in the group that had a titer of 1:100 AdHu26 VNAs at the time of immunization. In that group, the number of genital gag-specific $CD8^+$ T cells was higher than that in naive serum recipients.

To determine whether transfer of Ad-specific VNAs alters $CD4^+$ T cell responses, PBMCs and splenocytes were tested at 8 wk after immunization. We observed variation in the Ag-experienced ($CD44^+$) $CD4^+$ T cell responses in the blood after immunization with the different vectors. In comparison to the

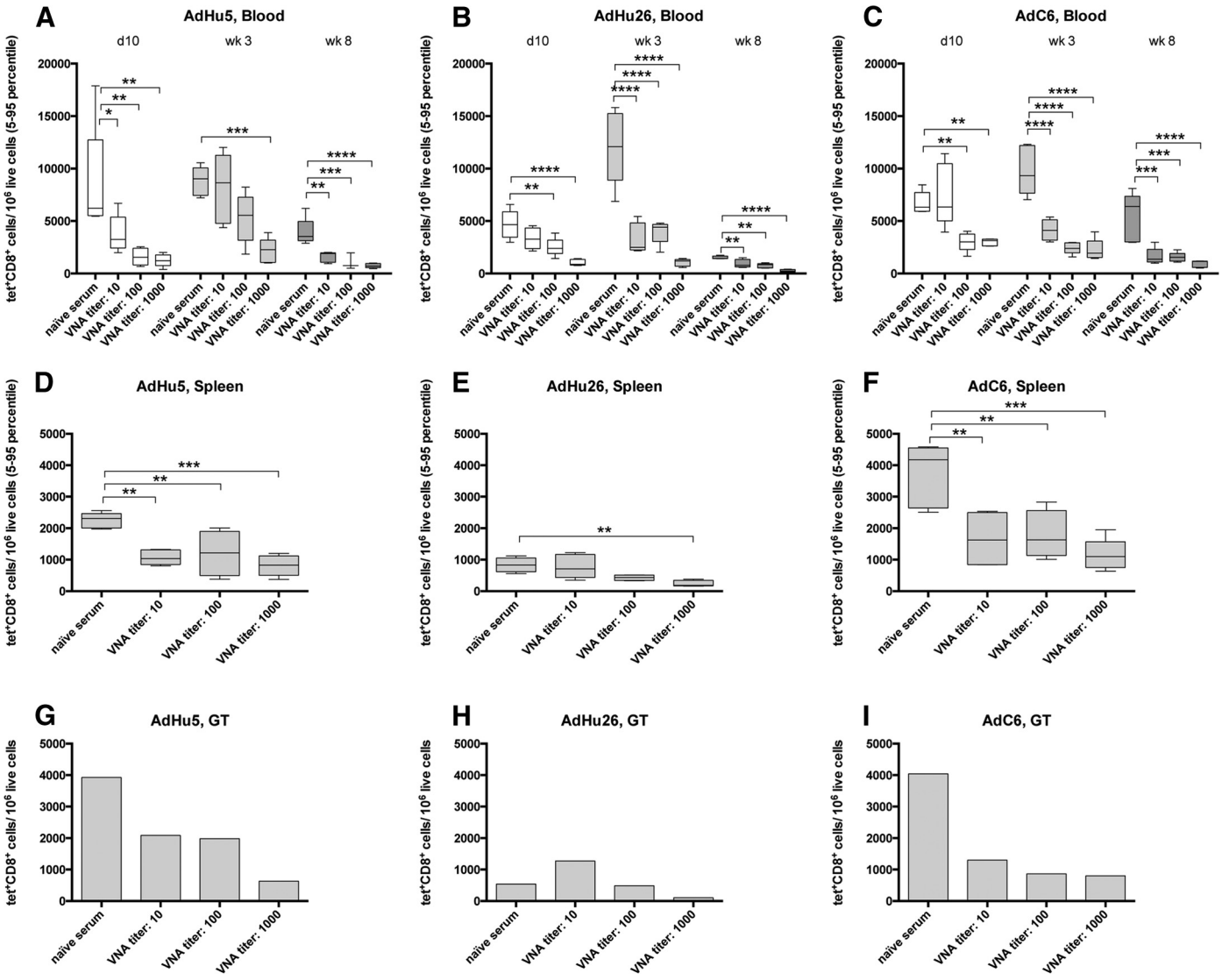

Figure 1. Passive transfer of Ad immune serum reduces transgene product–specific CD8$^+$ T cells induced following Ad vector immunization. Groups of female BALB/c mice (5 per group) were injected with pooled serum from donor mice that had been immunized twice with an Ad vector expressing rab.gp. Doses of serum were adjusted so that titers of Ad-specific VNAs measured from recipient mice 24 h later were 1:10, 1:100, or 1:1000. Control mice received a volume of serum from naive donor mice that equaled the highest volume of the immune serum. The mice were injected after serum transfer with 10^{10} vp of the Ad vector that was homologous to the Ad used to induce the transferred serum. Ad vectors expressed HIVgag. (A–C) Blood was collected on d 10 (open bars) and wk 3 (light gray bars) and 8 (dark gray bars), and the frequencies of gag-specific CD8$^+$ T cells were determined from the individual mice by staining with a specific tetramer. Data are expressed as the average number of tet$^+$CD8$^+$ cells/10^6 live PBMCs ± SD. Differences between the number of cells in immune vs. naive serum were determined by 1-way ANOVA with the Dunnett correction for type 1 errors: (A) AdHu5 d 10: 1:10, $P = 0.044$; 1:100, $P = 0.0037$; 1:1000, $P = 0.0025$. Wk 3: 1:1000, $P = 0.0006$. Wk 8: 1:10, $P = 0.0012$; 1:100, $P = 0.0007$; 1:1000, $P < 0.0001$. (B) AdHu26: d 10: 1:100, $P = 0.007$, 1:1000, $P < 0.0001$. Wk 3: all serum dilutions, $P < 0.0001$. Wk 8: 1:10, $P = 0.0012$; 1:100, $P = 0.0013$; 1:1000, $P < 0.0001$. (C) AdC6 d 10: 1:100, $P = 0.0073$; 1:1000, $P = 0.0068$. Wk 3: 1:10, $P < 0.0001$; 1:100, $P < 0.0001$; 1:1000, $P < 0.0001$. Wk 8: 1:10, $P = 0.0006$; 1:100, $P = 0.0004$; 1:1000, $P < 0.0001$. (D–F) Splenocytes were tested 8 wk after immunization, and the differences were obtained by 1-way ANOVA with the Dunnett correction for type 1 errors: (D) AdHu5 d 10: 1:10, $P = 0.001$; 1:100, $P = 0.0044$; 1:1000, $P = 0.0002$. (E) AdHu26: 1:1000, $P = 0.0061$. (F) AdC6: 1:10, $P = 0.0057$; 1:100, $P = 0.0086$; 1:1000, $P = 0.0006$. (G–I) Results of genital tract samples pooled from 5 mice, shown as the number of gag-specific CD8$^+$ T cells/10^6 live lymphocytes. *$P \leq$ 0.05-0.01; **$P <$ 0.01-0.001; ***$P \leq$ 0.001-0.0001; ****$P < 0.0001$. Brackets indicate the groups compared.

number of CD4$^+$ T cells found in the blood of naive mice, immunization with AdHu5 in the absence of vector-specific VNAs significantly increased CD4$^+$ T cell responses (**Fig. 2A**). However, priming in the presence of AdHu5-specific VNAs failed to increase CD4$^+$ T cell responses, which remained comparable to those in the naive mice. The exception to this finding was mice primed in the presence of the highest VNA dose, where the CD4$^+$ T cell responses became elevated above

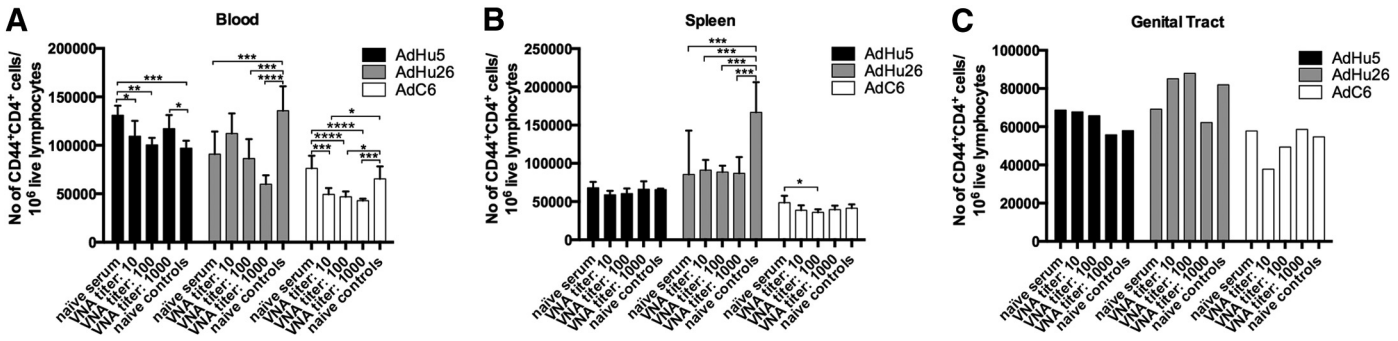

Figure 2. CD4$^+$ T cell responses in blood and spleen and recruitment to the genital tract. The relative number of CD44$^+$CD3$^+$CD8$^-$ cells isolated 8 wk after immunization are shown for (A) blood, the (B) spleen, and the (C) pooled genital tracts of the same mice as shown in Fig. 1. CD4$^+$ cells were identified by staining for CD8 and CD3 as cells that were CD3$^+$CD8$^-$. Data are the number of CD44$^+$CD4$^+$cells per 10^6 live cells ± SD. Significant differences between the number of lymphocytes from naive vs. immune sera and the number of lymphocytes from naive vs. immunized mice were determined by 1-way ANOVA with Dunnett correction for type 1 errors: (A) AdHu5: 1:10, $P = 0.025$; 1:100, $P = 0.0015$. AdC6: 1:10, $P = 0.0002$; 1:100, $P < 0.0001$; 1:1000, $P < 0.0001$. AdHu5 naive serum: $P = 0.0005$; 1:1000, $P = 0.0387$. AdHu26: naive serum: $P = 0.0082$; 1:100, $P = 0.0038$; 1:1000, $P < 0.0001$. AdC6: 1:10, $P = 0.0378$; 1:100, $P = 0.0148$; 1:1000, $P = 0.0030$. (B) AdC6: 1:100, $P = 0.0143$. AdHu26 naive serum, $P = 0.0035$, 1:10, $P = 0.0064$; 1:100, $P = 0.0049$; 1:1000, $P = 0.0041$. *$P \leq$ 0.05-0.01; **$P <$ 0.01-0.001; ***$P \leq$ 0.001-0.0001; ****$P < 0.0001$. Brackets indicate the groups compared.

baseline. In contrast, vaccination with AdHu26 significantly reduced the CD4$^+$ T cell responses in all groups except the mice primed in the presence of the lowest dose of AdHu26-specific VNAs. While immunization with AdC6 did not change the number of CD4$^+$ T cells in the blood, priming in the presence of AdC6-specific VNA, regardless of dose, reduced the CD4$^+$ T cell responses. The CD4$^+$ T cell responses in the spleen were largely unchanged after immunization with either AdHu5 or AdC6 (Fig. 2B). Splenic CD4$^+$ T cells in the AdC6-immunized mice were below those in the naive mice, regardless of transfer of AdC6-specific Abs.

In the STEP trial, individuals with moderate to high serum VNA titers to AdHu5 virus at the time of immunization showed a slight increase in HIV-1 acquisition rates [4]. Although the underlying causes of this heightened susceptibility remain unclear, increases in activated CD4$^+$ T cells serving as targets for HIV-1 may have contributed. We tested Ad-immunized mice that received the different serum preparations for a relative number of genital Ag-experienced (CD44$^+$) CD4$^+$ T cells in comparison to naive control mice. None of the vectors tested increased the relative number of activated CD4$^+$ T cells after vaccination, when compared to the number in the naive controls (Fig. 2C). Although we observed some variation in immune serum recipients (i.e., decreases in CD44$^+$CD4$^+$ cells in the AdC6 group that received low doses of serum vs. increases in the AdHu26 group), the overall differences were modest and within the range seen in the control mice.

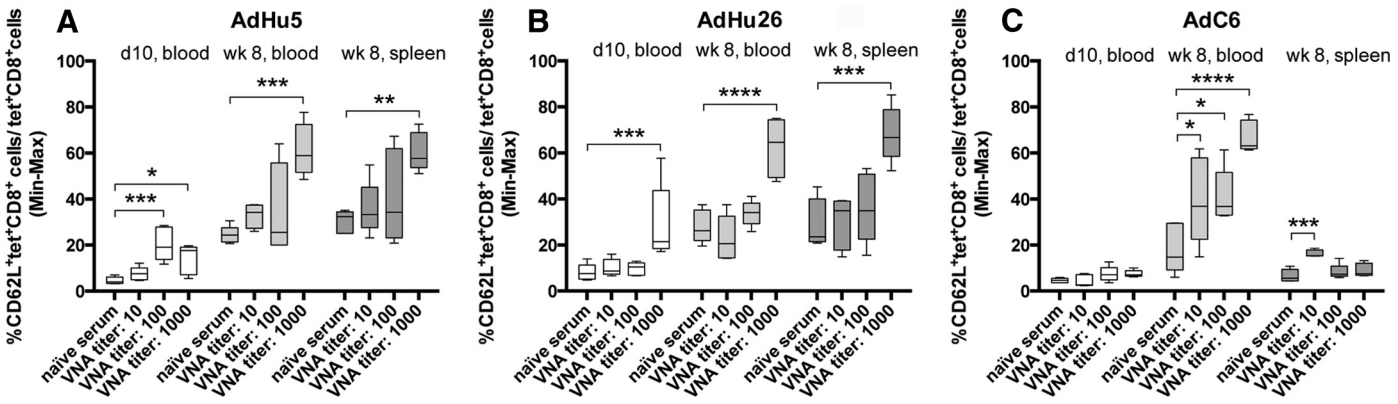

Figure 3. Pre-existing Ad-specific VNAs increase transgene product–specific CD62L$^+$CD8$^+$ T cells. Blood-derived, gag-specific CD8$^+$ T cells, identified by stains for CD3, CD8, and the gag-specific tetramer from the groups of 5 mice immunized as in Fig. 1 were tested for expression of CD62L 10 d (white) and 8 wk (light gray) after immunization. Splenocytes (dark gray) were tested 8 wk after immunization. Differences between frequencies in serum recipients vs. controls were determined by 1-way ANOVA with the Dunnett correction for type 1 errors. Blood: (A) AdHu5 d 10: 1:100, $P = 0.0005$; 1:1000, $P = 0.027$. Wk 8: 1:1000, $P = 0.0014$. (B) AdHu26 d 10: 1:1000, $P = 0.0046$. Wk 8: 1:1000, $P < 0.0001$. (C) AdC6 wk 8: 1:10, $P = 0.045$; 1:100, $P = 0.032$; 1:1000, $P < 0.0001$. Spleen (A) AdHu5 1:1000, $P = 0.0044$. (B) AdHu26: 1:1000, $P = 0.0008$. (C) AdC6: 1:10, $P = 0.0001$. *$P \leq$ 0.05-0.01; **$P <$ 0.01-0.001; ***$P \leq$ 0.001-0.0001; ****$P < 0.0001$. Brackets indicate the groups compared.

Effect of transfer of Ad-specific immune sera on memory formation of transgene product–specific CD8$^+$ T cell responses to a homologous Ad vector

Ad vectors persist at low levels in activated T cells in a transcriptionally active form and thereby maintain high frequencies of activated effector T cells with only a modest number of transgene product–specific T cells transitioning into memory [19]. The gag-specific CD8$^+$ T cells induced in our experiments were analyzed for the expression of CD62L, which can distinguish effector (CD62L^{low}) from the more resting memory (CD62L^{hi}) cells. Surprisingly, mice immunized with Ad vectors in the presence of Ad-specific VNAs showed increased frequencies of gag-specific CD62L$^+$CD8$^+$ T cells, for all 3 Ad vectors tested. The effect was observed as early as 10 d after immunization in AdHu5- and AdHu26-immunized mice that had received the highest dose of hyperimmune serum and became more pronounced over time (**Fig. 3**). In the AdC6-immunized groups, increases in gag-specific CD8$^+$ T cells with high CD62L expression did not become significant until wk 8 after vaccination. In this group, the serum effect was more pronounced on circulating than on splenic gag-specific CD8$^+$ T cells.

Increases in the frequency of gag-specific CD62L$^+$CD8$^+$ T cells could indicate that VNAs reduced the amount of virus that transfected the cells through neutralization of the vector, thereby affecting the level of viral persistence and allowing for increased memory formation. To address the argument that increased memory formation reflects a reduction in the numbers of infectious viral particles, we immunized mice with various doses of AdHu5 (10^8–10^{10}vp) and tested for percentages of CD62L$^+$ gag-specific CD8$^+$ T cells. There were significant differences in the magnitude of gag-specific CD8$^+$ T cell responses in blood when the mice were tested 5 and 8 wk after immunization (**Fig. 4A**); nevertheless, the percentages of CD62L$^+$ gag-specific CD8$^+$ cells within the responding CD8$^+$ T cell population remained comparable (Fig. 4B).

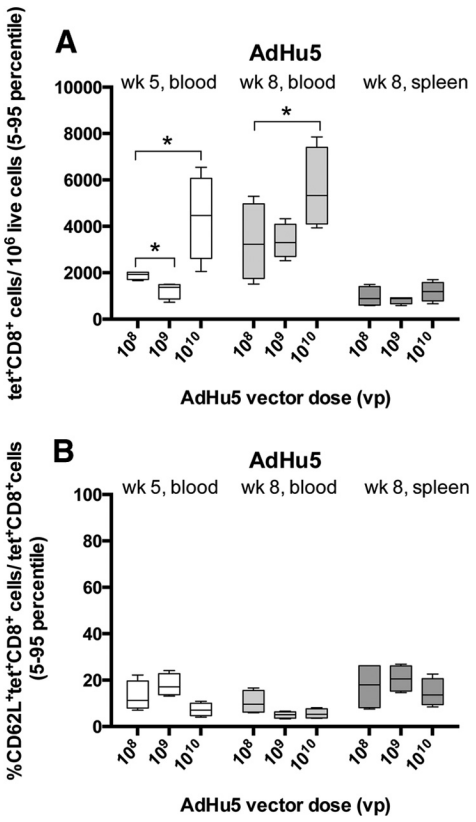

Figure 4. Low doses of Ad vector fail to promote increases in transgene product–specific CD62L$^+$CD8$^+$ T cells. Groups of 4 mice were vaccinated with 10^8, 10^9, or 10^{10} vp of AdHu5gag vector. (A) PBMCs were tested at wk 5 (white) and 8 (light gray), and splenocytes were tested at wk 8 (dark gray) for the number of gag-tet$^+$CD8$^+$ T cells/10^6 live lymphoid cells. The data were analyzed by ANOVA with uncorrected Fisher's LSD. Brackets indicate the groups compared. The following differences were significant in blood: wk 5: 10^8 vs. 10^9 vp, P = 0.012; 10^9 vs. 10^{10} vp, P = 0.047, and wk 8: 10^8 vs. 10^{10} vp, P = 0.033. (B) Tet$^+$CD8$^+$ T cells were tested for expression of CD62L, and the percentage of CD62L$^+$gag-specific CD8$^+$ T cells relative to all gag-specific CD8$^+$ T cells is shown. There were no significant differences in percentages of CD62L$^+$ cells. *$P \leq$ 0.05-0.01. Brackets indicate the groups compared.

Effect of transfer of Ad-specific immune serum on recall responses

Increased induction of memory CD8$^+$ T cells, with their higher proliferation potential when compared to more activated effector-like CD8$^+$ T cells, should result in augmented recall responses to antigenic challenge. Mice with an AdHu5 VNA titer of 1:1000 after serum transfer were vaccinated with 10^{10} vp of the AdHu5gag vector, and blood was collected 8 wk later for evaluation of gag-specific CD8$^+$ T cell responses. As expected, pre-existing immunity resulted in a significant reduction in the number of gag-specific CD8$^+$ T cells (**Fig. 5A**). Gag-specific CD8$^+$ T cells were analyzed for expression of differentiation markers: CD62L; KLRG-1, a marker for terminal differentiation [20]; CD127, a marker for cells destined to enter the memory T cell pool [21]; and CCR7, a chemokine receptor that promotes lymph node homing of resting T cells [22] (Fig. 5F). As in the previous experiment, the transferred immune serum induced a shift toward increased percentages of gag-specific CD62L$^+$CD8$^+$ T cells in combination with decreases in KLRG-1$^+$ cells (Fig. 5B). There were trends toward higher percentages of CD127$^+$ or CCR7$^+$ cells in immune serum recipients that did not reach significance after type 1 error correction. Shortly after collection of blood samples, the mice received booster injections of 10^{10} vp of an AdC7gag vector, and the T cell responses were analyzed in blood collected 10 d later. The booster immunization caused robust increases in circulating gag-specific CD8$^+$ T cells in all mice (Fig. 5A). The number of gag-specific CD8$^+$ T cells increased on average 14-fold in the group that had been treated with naive serum at the time of priming and 35-fold in the group that had received hyperimmune serum, a substantial difference (P=0.008). After the boost, expression of differentiation markers on gag-specific CD8$^+$ T cells was largely comparable, with the exception of KLRG1, which was expressed on a lower percentage of gag-specific CD8$^+$ T cells from mice with pre-existing AdHu5-specific VNAs (Fig. 5C).

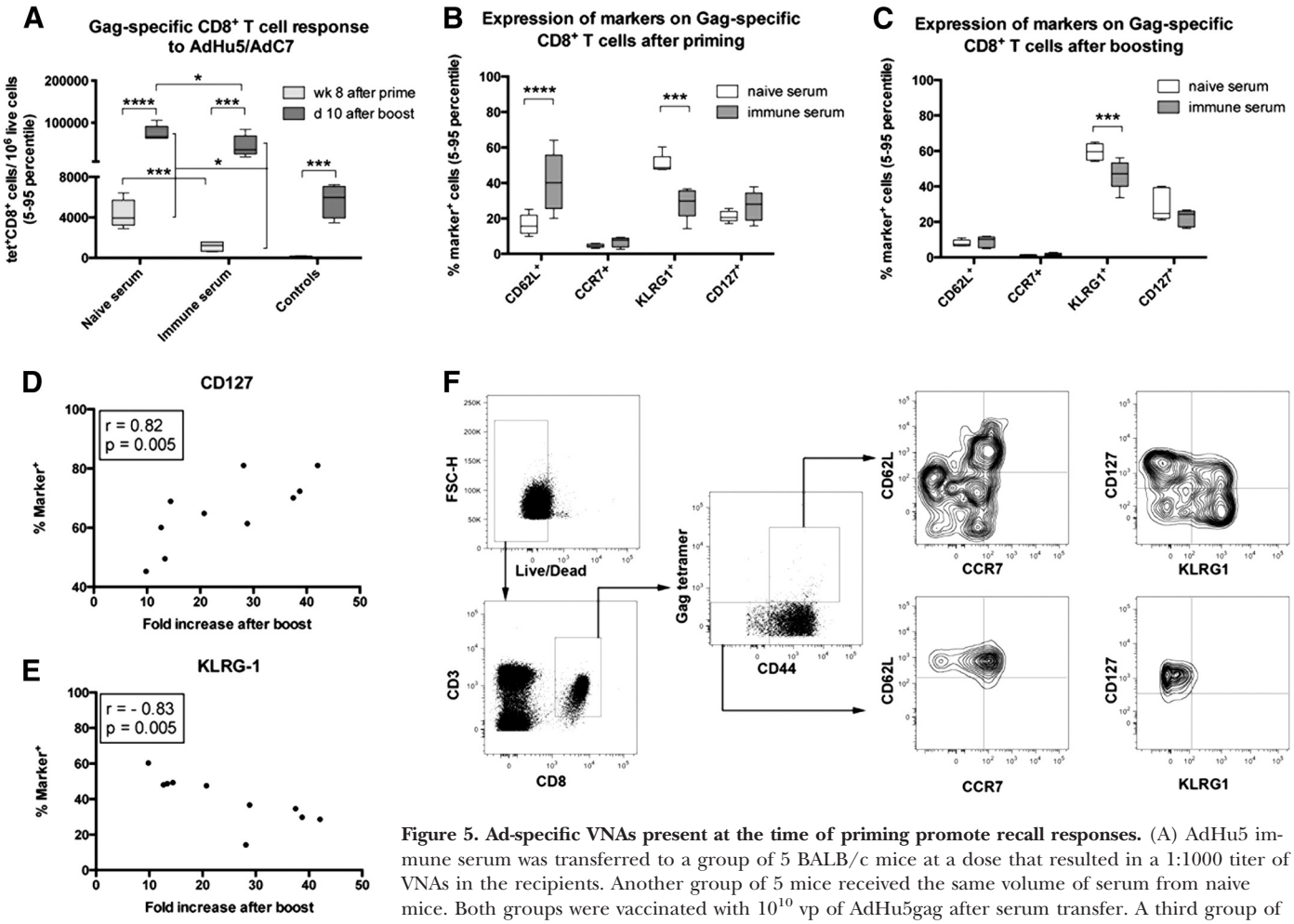

Figure 5. Ad-specific VNAs present at the time of priming promote recall responses. (A) AdHu5 immune serum was transferred to a group of 5 BALB/c mice at a dose that resulted in a 1:1000 titer of VNAs in the recipients. Another group of 5 mice received the same volume of serum from naive mice. Both groups were vaccinated with 10^{10} vp of AdHu5gag after serum transfer. A third group of 5 mice was left untreated (controls). Eight weeks later, the blood was collected and gag-specific $CD8^+$ T cells were measured (light gray). All mice were vaccinated the following day with 10^{10} vp of an AdC7gag vector. Gag-specific $CD8^+$ T cell responses were measured in the blood 10 d later (dark gray). Shown is the number of gag-specific $CD8^+$ T cells stained of the tetramer/10^6 live lymphoid cells (min to max) ± SD. Differences between the 2 groups of serum recipients were determined by *t* test. $P = 0.0044$, recipients of immune vs. naive serum after priming. Differences in the same cohort, comparing data after priming with those after the boost. Naive serum recipients, $P < 0.0001$; immune serum recipients, $P = 0.0043$. Fold increases in the number of gag-specific $CD8^+$ T cells were calculated (P=0.011, immune vs. naive serum). Controls (no priming, AdHu5 at the time of boost), $P = 0.0001$. (B) Percentage of gag-specific $CD8^+$ T cells tested 8 wk after immunization for expression of CD62L, CCR7, KLRG1, and CD127. CD62L, $P < 0.0001$; KLRG1, $P = 0.00012$; multiple *t* tests with the Holm-Šídák correction. (C) Percentages of gag-specific $CD8^+$ T cells positive for the indicated markers/all $CD8^+$ T cells at 10 d after the boost in mice that received immune or naive serum before priming. Only KLRG1 was differentially expressed ($P = 0.0004$). $^*P \leq 0.05$-0.01; $^{***}P \leq 0.001$-0.0001; $^{****}P < 0.0001$; multiple *t* tests with the Holm-Šídák correction. Brackets indicate the groups compared. Correlations between the fold increases after the boost and the percentage of gag-specific $CD8^+$ T cells positive for (D) CD127 and (E) KRLG-1 are shown. (F) Gating strategy for a representative sample. After lymphocytes were identified based on forward scatter and side scatter, the doublets were excluded (not shown). Live cells were identified based on exclusion by live/dead staining. $CD8^+$ T cells were gated ($CD3^+CD8^+$). The subsequent gates were designed to isolate gag-specific $CD8^+$ T cells (tet^+CD44^+) and naive cells (tet^-CD44^{low}) as an internal control. Each population was then gated on CD62L and CCR7 or CD127 and KLRG1. Gates were determined based on the naive cells' expression of each marker.

The fold increases in the number of gag-specific $CD8^+$ T cells correlated (according to Spearman) with several of the markers expressed at 8 wk after priming. Positive correlations were seen for frequencies of cells expressing CD127 (r=0.821, P=0.005; Fig. 5D), whereas inverse correlations were observed for cells expressing KLRG1 (r=−0.83, P=0.0047; Fig. 5E). Other correlations, such as for CD62L (r=0.709, P=0.027), were not significant after the Bonferroni correction for type 1 error. Overall, these results confirm that priming in the presence of vector-specific VNAs increases memory formation and thereby recalls responses of transgene product–specific $CD8^+$ T cells.

Effect of transfer of Ad-specific immune sera on vector transduction rates and persistence

VNAs to the Ad vector are expected to neutralize the vaccine and thereby reduce the vectors' ability to transduce cells and express the transgene product. This effect was formally tested by injecting mice with intermediate doses of naive and AdHu5 immune sera, with the latter inducing titers of ~1:100 in re-

cipient mice. The mice were then injected with the AdHu5gag vector. Three days later, muscle tissue from the injection sites, spleens, and lymph nodes were harvested, and DNA was isolated. An aliquot of the DNA was used in qPCR to determine relative copy numbers of gag sequences. As shown in **Fig. 6**, there was a clear trend of higher gag copy numbers in mice that had been injected with the naive serum. Nevertheless, differences between the naive and immune serum recipients failed to reach significance. A second cohort of mice was tested 8 wk after immunization for the presence of vector-derived DNA sequences in spleens and lymph nodes. qPCR showed weak amplification of only 2 samples (1 from spleen and 1 from lymph nodes) from naive serum recipients. We therefore used a more sensitive nested PCR [19] for the analysis. A nested PCR does not quantify transcript copies but, due to its very high sensitivity, gives a reliable picture of the continued presence of persisting vectors. As shown in **Fig. 7A–D**, 4 of 4 mice that had received the naive serum had residual AdHu5gag vector–derived DNA in spleens and lymph nodes, although the signal was rather weak in 1 of the spleen samples. Immune serum recipients showed vector-derived transcripts persisting in 3 of 4 lymph node samples and 4 of 4 spleen samples, although again the signal was weak in 1 of the lymph node samples. In our study of the kinetics of vector persistence [19], we observed fluctuations in the samples tested (i.e., mice would score negative at some time points, but positive at later time points). The lack of amplification in 1 sample thus does not necessarily reflect a lack of persistence in this animal, especially as the same animals showed positive amplification of the lymph node sample. Overall, these results show that, although Ad vector–specific immune sera cause a slight reduction in the initial transduction rates, the vectors persist in the transduced cells.

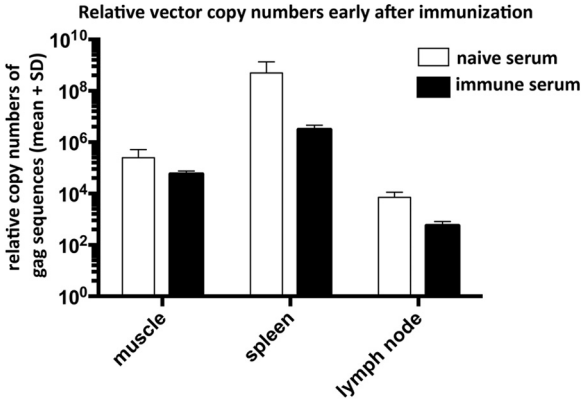

Figure 6. Effect of pre-existing Ad-specific Abs on AdH5gag vector transduction rates. Groups of 4 mice that had received naive or AdHu5 immune serum (titer, ~1:100) before immunization with the AdHu5gag vector (10^{10} vp/mouse) were euthanized 3 days after vector injection. Muscles, spleens, and lymph nodes were isolated. DNA was isolated from 25 mg of muscle or 5×10^6 lymphocytes from spleens and lymph nodes. Aliquots of the DNA (2 μL/50 μL) were then amplified by qPCR with gag- and GAPDH- specific primers. Shown is the mean relative gag copy numbers ± SD in 10^6 lymphocytes or 1 mg of muscle tissue. C_T values obtained for gag were first normalized to C_T values obtained for GAPDH. A standard curve based on amplification of serially diluted gag plasmid was used to estimate the copy numbers. Data were analyzed by multiple *t* tests with the Holm-Šídák correction. Analysis showed no significant differences between the 2 groups.

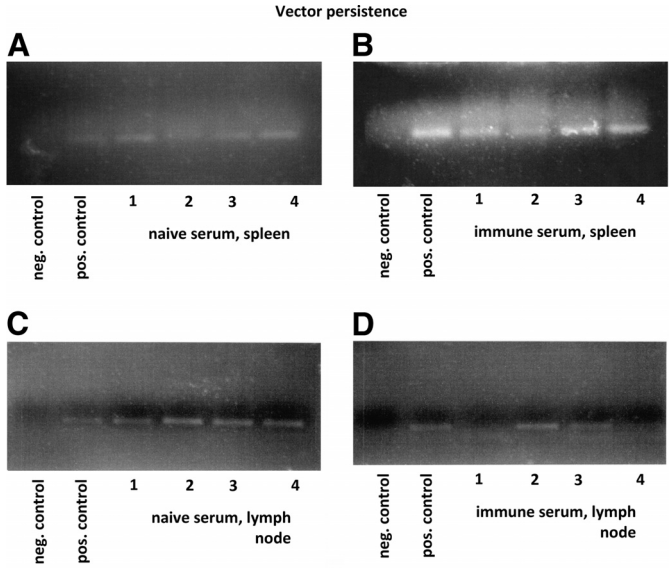

Figure 7. Effect of pre-existing Ad-specific Abs on AdHu5gag vector persistence. Mice (n=4 per group) treated as described in Fig. 7 were euthanized 8 wk after vector injection. Lymphocytes were isolated from spleens and lymph nodes, and DNA was isolated from 5×10^6 lymphocytes per sample. An aliquot of this reaction (2 μL/50 μL) was amplified by nested PCR based on gag-specific primers. None of the samples showed amplicons after the first PCR. Amplicons obtained after the nested PCR were separated by gel electrophoresis. Amplicons of the experimental samples are shown, as well as negative (water) and positive (gag plasmid) controls that were subjected in parallel to the experimental conditions for both rounds of PCR. Additional controls included lymphocytes from naive mice and water used only in the second PCR (not shown). A molecular weight marker (not shown) was included in the electrophoresis gel, to confirm that the amplicons had the expected size. Results are shown for splenocytes of naive (A) and immune (B) serum recipients and for lymph node lymphocytes of naive (C) and immune (D) serum recipients. Arrows: amplicons.

Effect of transfer of Ad-specific immune sera on the magnitude of transgene product–specific CD8$^+$ T cell responses to a heterologous Ad vector

Ad viruses induce serotype-specific VNAs that are largely directed against the highly variable loops on the globular head of the viral hexon [23]. Other sequences that may provide targets for Abs, such as those forming the hexon stalk, are more conserved among distinct serotypes. To assess whether transfer of an Ad-specific immune serum influences vaccination with a heterologous Ad vector, we first assessed the sera used in transfer experiments for reactivity to AdHu5, AdHu26, AdC6, and AdC7 vectors by ELISAs. AdC7 vectors are derived from SAdV24 virus, which belongs to a different serotype than SAdV23 [14], the basis for AdC6 vectors. As shown in **Fig. 8A**, sera preferentially bound to the homologous virus, although there was clear cross-reactivity with other serotypes for AdHu26 and AdC6 immune sera.

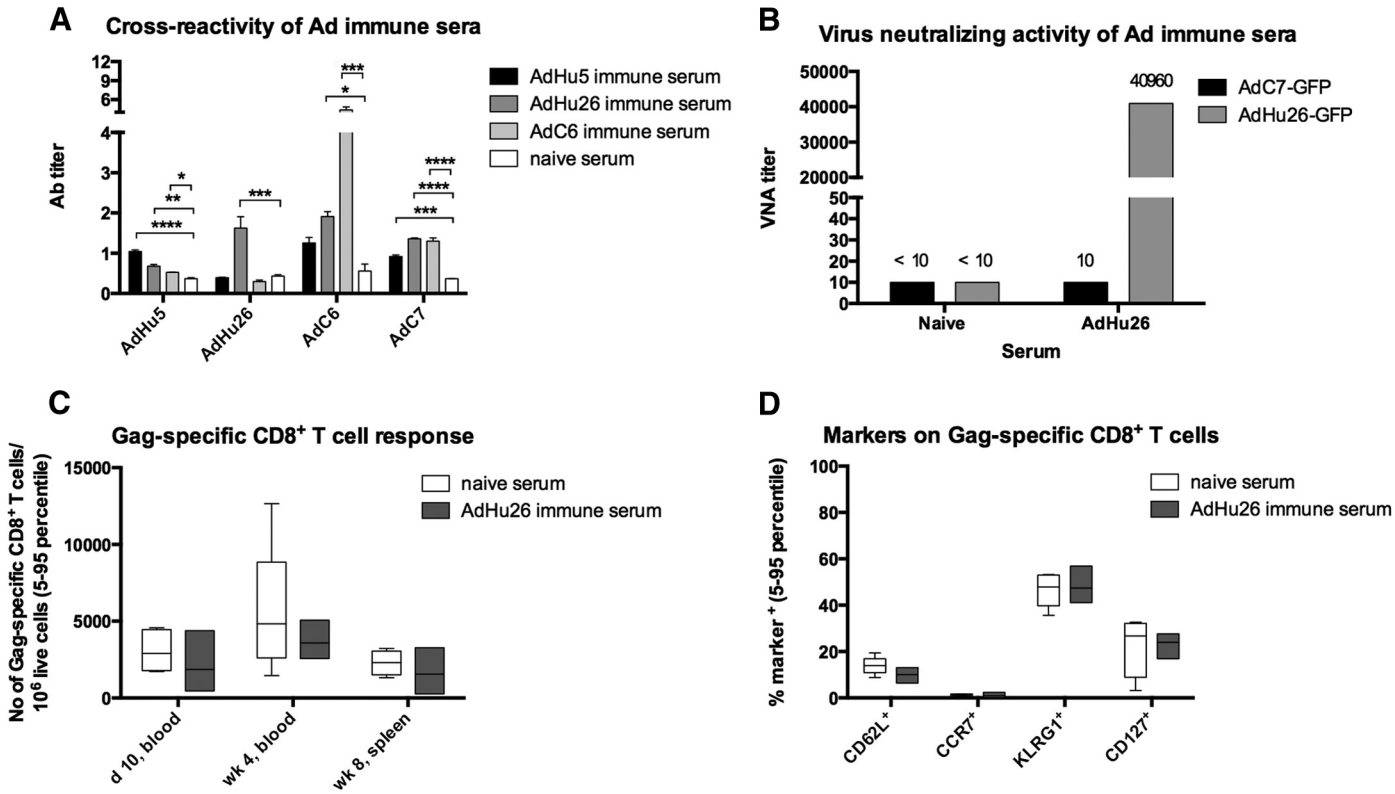

Figure 8. Effect of pre-existing Ad-specific Abs on transgene product–specific CD8$^+$ T cells to a heterologous Ad vector. (A) Binding of the transferred sera to different Ad serotypes, as shown in the key within the figure. Titers were calculated by determining the area under the curve of adsorbence data obtained by serially diluted sera. ELISAs were conducted in duplicate wells and results are expressed as the mean ± SD. Differences between the titers of immune and naive sera were calculated by ANOVA with the Dunnett correction. The following sera showed significant reactivity with the following vectors. (A) AdHu5 serum: on AdHu5 vector, $P < 0.00001$, and on AdC7 vector, $P = 0.0006$. AdHu26 serum: on AdHu5 vector, $P = 0.0013$; on AdHu26 vector, $P = 0.0008$; on AdC6 vector, $P = 0.0132$; and on AdC7 vector, $P < 0.0001$. AdC6 serum: on AdHu5 vector, $P = 0.016$; on AdC6 vector, $P = 0.0003$; and on AdC7 vector, $P < 0.0001$. (B) Analysis of naive BALB/c sera and AdHu26 hyperimmune sera for neutralization of AdHu26 or AdC7. Sera were tested for virus-neutralizing Abs against AdC7-GFP and AdHu26-GFP in a VNA assay. Titers of 10 and lower were considered nonneutralizing. Groups of 5 mice were injected with a dose of AdHu26 immune serum that resulted in a VNA titer of 1:1000 or an equal dose of serum from naive mice. Mice were then immunized with AdC7gag. (C) Gag-specific CD8$^+$ T cell responses tested from blood at 10 d and 4 wk and from lymph nodes and spleens at 8 wk. (D) Percentage of gag-specific CD8$^+$ T cells that were positive for the indicated markers. *$P \leq 0.05$-0.01; **$P < 0.01$-0.001; ***$P \leq 0.001$-0.0001; ****$P < 0.0001$. Brackets indicate the groups compared.

AdHu26 VNAs are common in humans residing in developing countries [16] and transfer of these VNAs strongly reduced transgene product–specific CD8$^+$ T cell responses after AdHu26gag vaccination (Fig. 1), causing a pronounced increase in gag-specific CD62L$^+$CD8$^+$ T cells as early as 3 wk after vaccination (Fig. 3). In addition, binding Abs induced by AdHu26 immunization showed strong cross-reactivity with AdC7 vectors (Fig. 8A). Although AdHu26-specific Abs showed cross-reactive binding with AdC7 vector, they did not neutralize the AdC7 vector, as shown in Fig. 8B. Therefore, we next assessed whether transfer of an AdHu26-immune serum affects CD8$^+$ T cell responses and memory formation after AdC7gag vaccination. Mice with a high dose of transferred AdHu26 immune serum that induced VNA titers of 1:1000 or the same dose of the control serum were immunized with AdC7gag. T cell responses were measured in blood collected 10 d and 4 wk later and in spleens harvested 8 wk after vaccination. Results clearly show that the AdHu26-immune serum did not reduce the gag-specific CD8$^+$ T cell responses elicited by the AdC7gag vector (Fig. 8C). Key markers that were differentially expressed in the presence of a homologous immune serum (Fig. 3) were analyzed at 4 wk on gag-specific CD8$^+$ T cells from blood. Transfer of immune serum to AdHu26 did not modify expression of CD62L, CD127, or KLRG1 on AdC7-induced, transgene product–specific CD8$^+$ T cells (Fig. 8D), indicating that the effects seen within a homologous vector system (Fig. 3) were driven by serotype-specific VNAs.

DISCUSSION

The current study was conducted in mice to assess whether and how Ad-specific Abs that are present at the time of immunization affect the transgene product–specific CD8$^+$ T cells elicited by different Ad vector serotypes. The study had several goals. We wanted to assess whether VNAs to different Ad sero-

types have equal effects on the magnitude of the transgene product–specific $CD8^+$ T cell responses induced by homologous vectors. We tested to learn whether the Ad-specific Abs present at the time of priming change the course of transgene product–specific $CD8^+$ T cell responses. Finally, we aimed to establish whether Ad virus–specific Abs affect the magnitude or differentiation of transgene product–specific $CD8^+$ T cells induced by a serologically distinct Ad vector.

In our study, VNAs reduced the induction of transgene-specific $CD8^+$ T cells. There were differences in the susceptibility and kinetics of the 3 Ad serotypes to VNA-mediated inhibition. The potency of stimulated T cell responses was subtle overall, however some variation was observed. Although Ad vectors induce strong inflammatory responses [24] and elicit transgene product–specific $CD8^+$ T cells that readily migrate to the genital tract [18], none of the Ad vectors, in the presence or absence of pre-existing Ad-specific VNAs, increased the number of activated genital $CD4^+$ cells, which could serve as targets for HIV-1 acquisition.

Unexpectedly, VNAs specific to the immunizing vector promoted transition of transgene product–specific $CD8^+$ T cells into the memory pool. Ad vectors persist at low levels in activated T cells where they remain transcriptionally active [19], and this continued stimulation maintains transgene product–specific $CD8^+$ T cells in an activated effector stage with only limited differentiation into central memory. Increased memory formation in the presence of vector specific VNAs, as evidenced by increased expression of CD62L and enhanced expansion of Ad vector-induced $CD8^+$ T cells after in vivo re-exposure to Ag, may indicate that the limited dose of vector that escaped neutralization did not present sufficient Ag to effectively drive differentiation of most of the responding $CD8^+$ T cells into the effector memory pool. In fact, previous studies showed that antigenic load is one of the factors that determine the fate of $CD8^+$ T cells after their stimulation [25]. The increased memory formation seen in the presence of the VNAs was not replicated by a 100-fold reduction of the dose of Ad vectors in animals without pre-existing VNAs. This result makes it unlikely that VNAs drive memory formation solely through reduction of the infectious vector load, which decreases the antigenic load, but rather suggests that VNAs more selectively inhibit transduction of T cells, which serve as reservoirs for persisting Ad vectors [19, 26]. This possibility was not confirmed experimentally. Although neutralizing Abs to the Ad vector caused a slight, albeit insignificant, reduction in cell-associated vector genome copies early after injection of the vector, the recipients of immune sera showed persistence of vector in lymphatic tissues, suggesting that mechanisms other than lack of continued presence of the Ag contribute to the improved differentiation of $CD8^+$ T cells into the central memory pool. Cross-reactive nonneutralizing Abs to the Ad vectors did not reduce the transgene product–specific $CD8^+$ T cell responses induced by a different serotype of Ad vector, nor did they cause detectable increases in memory formation.

Could an increased expansion of HIV-1-specific $CD8^+$ T cells after an antigenic challenge increase the risk of HIV-1 infection? In a preclinical SIV nonhuman primate infection model, protection induced by a highly immunogenic rhesus macaque CMV vector was linked to effector memory $CD8^+$ T cells [27], whereas another study implied a role for central memory $CD8^+$ T cells in resistance to HIV-1 [28]. One would assume that, during any viral infection, effector memory $CD8^+$ T cells present at the port of viral entry are key to the rapid elimination of infected cells, whereas central memory $CD8^+$ cells, which need to expand for several days before they gain effector functions, are crucial to controlling breakthrough infections [29]. Although it seems unlikely that an increased potential for Ag-driven expansion of specific $CD8^+$ T cells would increase the risk of infection, one could argue that an enhanced $CD8^+$ T cell recall response after infection concomitantly affects responses and trafficking patterns of $CD4^+$ T cells.

AUTHORSHIP

J.C.S. and L.H.H. conducted the experiments, analyzed the data, and wrote the manuscript. A.B. conducted the PCR studies. H.C.E. designed the experiments, analyzed the data and wrote the manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

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KEY WORDS:
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