

RESEARCH ARTICLE

Vaccination with helper-dependent adenovirus enhances the generation of transgene-specific CTL

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Recombinant adenoviral vectors (AdV) have been used experimentally as vaccines to present antigenic transgenes *in vivo*. However, administration of first-generation vectors (FG-AdV) is often limited by their induction of antiviral immunity. To address this limitation, helper-dependent vectors (HD-AdV) were developed that lack viral coding regions. While the administration of HD-AdV results in long-term gene expression *in vivo*, their utility as immunogens has never been examined. Direct vaccination with 10⁸ blue-forming units (BFU) of HD-AdV injected into C57BL/6 mice lead to superior transgene-specific CTL and antibody responses when compared to the same amount of a FG-AdV. The antibody responses to viral antigens were high in response to both the vectors. As a mechanism to reduce viral exposure, dendritic cells (DC) were transduced with HD-AdV *in vitro* and then used as a cell-based vaccine. DC

transduced with HD-AdV expressed higher levels of transgene-specific mRNA and up to 1200-fold higher levels of transgene protein than did DC transduced with a FG-AdV. In addition, HD-AdV-transduced DC stimulated superior transgene-specific CTL responses when administered *in vivo*, an effect that was further enhanced by maturing the DC with LPS prior to administration. In contrast to direct immunization with HD-AdV, vaccination with HD-AdV-transduced DC was associated with limited antibody responses against the AdV. We conclude that HD-AdV stimulates superior transgene-specific immune responses when compared to a FG-AdV, and that immunization with a DC-based vaccine maintains this efficacy while limiting antiviral reactivity.

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Introduction

Adenoviral vectors (AdV) deliver genes to both dividing and nondividing cells and have been widely tested as agents for gene therapy.¹ First-generation AdV (FG-AdV), such as E-1 deleted vectors, are replication deficient, but still expresses low levels of viral genes after infection.^{2,3} Their repeated administration is limited by rapid immune clearance of virus and virus-infected cells, thereby limiting transgene expression and producing inflammatory responses *in vivo*. Helper-dependent adenoviruses (HD-AdV) were developed in response to this problem and are termed 'gutless' vectors because they lack all viral-coding regions. When compared to FG-AdV, the administration of HD-AdV to animals results in potent and long-term gene expression *in vivo*.^{5–6} It was recently reported that HD-AdV can be used to transduce human DC and produce very high levels of transgene expression.⁷ However, DC transduced in this manner still evoked antiviral immune responses *in vitro*.⁷ Other than this limited information, little is known about the

potential for using HD-AdV as a vaccine agent, or their capacity to stimulate transgene-specific *versus* antiviral immune responses.

The immunogenicity of HD-AdV was therefore evaluated using two different vaccine strategies. In the first, mice were directly immunized with either HD-AdV or FG-AdV, both expressing the same *LacZ* transgene, and monitored for the induction of β -gal-specific CTL, β -gal-specific antibody, and anti-AdV antibody. Immunization with HD-AdV resulted in the induction of superior CTL and anti- β -gal antibody responses when compared to FG-AdV, but an equally potent anti-AdV response. In order to limit direct viral exposure, murine DC were transduced with either HD-AdV or FG-AdV *in vitro* and then administered to mice as a cell-based vaccine. HD-AdV was extremely effective at expressing transgene antigen in DC and stimulated superior β -gal-specific CTL responses when transduced DC were injected into mice. Maturation of transduced DC with LPS or transduction with a higher MOI further enhanced the CTL response. In contrast to direct immunization with virus, this cell-based approach stimulated only minor antibody responses to either β -gal or AdV. While still capable of stimulating anti-AdV immune responses, it appears that HD-AdV vectors express very high levels of transgene antigens and can provide an important approach for stimulating transgene-specific immunity.

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Results

HD-AdV induces higher transgene-specific CTL responses than FG-AdV

Relative vaccine potency was evaluated by immunizing mice with 10^8 BFU of either HD-AdV/ β -gal or FG-AdV/ β -gal and their spleen cells harvested 1 week later to measure the induction of β -gal-specific CTL (Figure 1a). The lysis of E22 targets, which express β -gal, was compared to the lysis of EL-4 cells, the parental line lacking β -gal. Nonspecific lysis of EL-4 was routinely <20% even at the highest E:T ratios (not shown). Vaccination with the HD-AdV/ β -gal virus generated a significantly higher β -gal-specific CTL response under all experimental conditions. A single immunization with HD-AdV/ β -gal resulted in $57.5 \pm 2.3\%$ lysis of E22 targets at a 60:1 E:T ratio, as compared to only a $28.3 \pm 0.5\%$ lysis induced by FG-AdV/ β -gal (Figure 1a) and $20.1 \pm 2.4\%$ lysis by spleen cells from nonimmunized mice ($P < 0.01$). CTL responses were boosted in mice that were immunized twice, but the relative activity of spleen cells obtained from HD-AdV/ β -gal-treated mice remained superior to that produced by FG-AdV/ β -gal (Figure 1b).

Both FG-AdV and HD-AdV stimulate potent anti-AdV antibody responses

The administration of FG-AdV is known to stimulate anti-AdV antibodies, which may neutralize subsequent viral injections.^{2,3} While both FG-AdV and HD-AdV are packaged within the same viral capsid (AdV type 5), HD-AdV are known for their ability to produce lasting *in vivo* expression even with repeated administration.^{8,9} Both viruses were therefore evaluated for their ability to

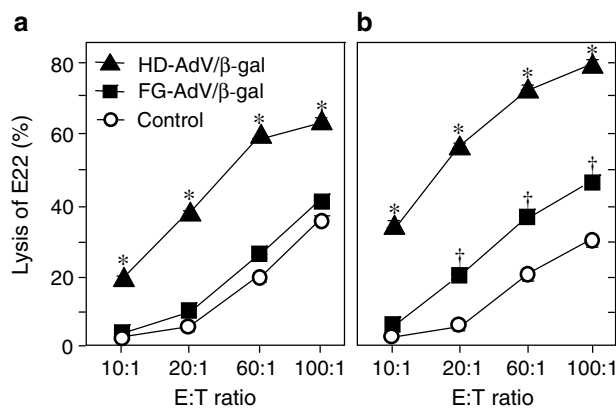


Figure 1 Immunization with HD-AdV/ β -gal induced a more potent transgene-specific CTL response than immunization with FG-AdV/ β -gal. Splenocytes were isolated from mice immunized once (a) or twice (b) at 7 day intervals with PBS alone (control) or 1×10^8 BFU of either HD-AdV/ β -gal or FG-AdV/ β -gal and stimulated for 5 days *in vitro* with irradiated E-22 cells. Activated T cells were then cocultured for 4 h in a standard [⁵¹Cr]-release assay with either E-22 target cells, expressing β -gal, or the parental cell line EL-4, not expressing β -gal, and the percent tumor lysis determined. Lysis of EL-4 targets was < 20% in all cases and not shown. Data represent the mean \pm s.e. of triplicate determinations for each group (2 mice/group). * $P < 0.001$ comparing splenocytes from HD-AdV/ β -gal immunized mice to control splenocytes or splenocytes from FG-AdV/ β -gal immunized mice. † $P < 0.01$ comparing splenocytes from FG-AdV/ β -gal immunized mice to control splenocytes. Representative data from one of four experiments.

induce anticapsid antibody responses in our vaccine model. A single immunization employing either HD-AdV/ β -gal or FG-AdV/ β -gal resulted in the production of high-titer anti-AdV responses that were similar for both vectors (not shown). When mice were immunized a second time with HD-AdV/ β -gal or FG-AdV/ β -gal, a significantly higher anti-AdV titer was observed in animals treated with FG-AdV, but this difference was relatively minor compared to the overall magnitude of the response (Figure 2a). As such, the deletion of viral-coding regions in the HD-AdV did not appear to appreciably reduce the anti-AdV response, consistent with reports that viral capsid antigens, which are shared by the two vectors, act as the major antigens.⁷

Anti-adenoviral antibody responses occur in response to both vectors, while anti- β -gal-specific antibody responses occur only in response to HD-AdV/ β -gal and are primarily of the IgG2a isotype

While both vectors induce similarly potent anti-AdV antibody responses, HD-AdV/ β -gal induced a strong anti- β -gal antibody response while FG-AdV did not (Figure 2b). In order to further characterize the nature of this antibody response against the AdV and β -gal antigens, specific antibodies belonging to the IgG2a isotype, associated with T helper 1 (Th1) responses, and IgG1 isotype, associated with Th2 responses, were independently measured.¹⁰ Isotype responses directed against AdV antigens belonged to both the IgG1 (Figure 3a) and IgG2a (Figure 3b) subclasses, although IgG2a responses frequently predominated. Immunization with HD-AdV/ β -gal always produced slightly lower antibody responses to the viral antigen than immunization with FG-AdV/ β -gal. However, direct immunization with FG-AdV/ β -gal failed to stimulate anti- β -gal antibodies of either isotype (Figure 3c). In contrast, immunization with

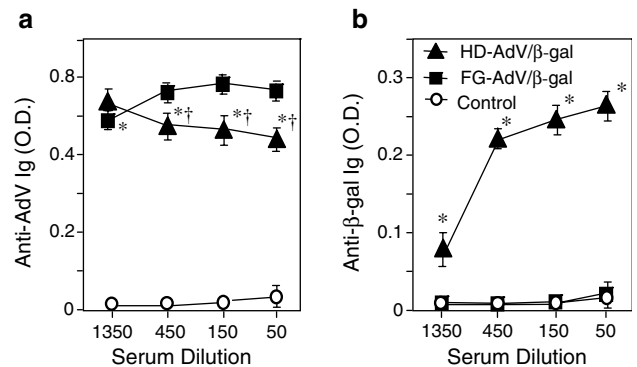


Figure 2 Both HD-AdV/ β -gal and FG-AdV/ β -gal induce similar anti-adenoviral antibody (Ig) responses (a), but a higher β -gal transgene-specific antibody (Ig) response is induced by immunization with HD-AdV/ β -gal than with FG-AdV/ β -gal (b). Mice were immunized twice at 7 day intervals with 1×10^8 BFU of either HD-AdV/ β -gal or FG-AdV/ β -gal or PBS alone (control). At 7 days after the last immunization, serum was collected and analyzed for the presence of adenovirus-specific total antibody (Ig) by ELISA using irradiated FG-AdV/RR5 as the immobilized antigen. Data represent the mean \pm s.e. optical density (OD) measured, as a measure of relative concentration, for serial dilution of sera pooled from two animals per group, assayed in triplicate. * $P < 0.001$ comparing serum from immunized mice to that from control mice. † $P < 0.01$ comparing serum from FG-AdV/ β -gal immunized mice to serum from HD-AdV/ β -gal immunized mice. Representative data from one of four experiments.

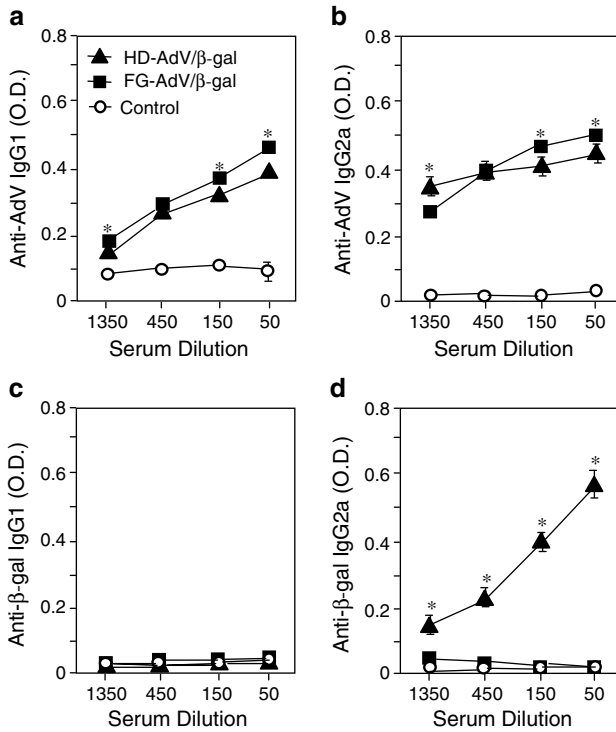


Figure 3 Antiadenoviral antibody responses belonging to both the IgG1 (a) and IgG2a (b) isotypes occur in response to both FG-AdV/β-gal and HD-AdV/β-gal, while anti-β-gal-specific antibody responses occur only in response to HD-AdV/β-gal and are primarily of the IgG2a isotype (c, d). Mice were immunized two times with HD-AdV/β-gal and FG-AdV/β-gal as described in Figure 2. At 7 days after last administration of 1×10^8 BFU of HD-AdV/β-gal and FG-AdV/β-gal sera from mice were analyzed for the presence of AdV and β-gal-specific IgG1 and IgG2a antibodies by ELISA using irradiated FG-AdV/RR5 as the immobilized antigen and purified β-gal protein as target antigen. Data represent the mean \pm s.e. optical density (OD) measured for serial dilution of sera pooled from two animals per group, assayed in triplicate. * $P < 0.01$ comparing serum from immunized mice to that from FG-AdV/β-gal immunized mice and control mice sera. Representative data from one of four experiments.

HD-AdV/β-gal produced a marked and selective increase of transgene-specific antibodies belonging to the IgG2a subclass (Figure 3d).

DC transduced with HD-AdV/β-gal express higher levels of β-gal protein and mRNA

We hypothesized that the superior CTL and antibody responses induced by HD-AdV/β-gal were related to the capacity for this vector to express high levels of transgene, resulting in efficient antigen loading into DC. To evaluate this hypothesis, both HD-AdV/β-gal and FG-AdV/β-gal were analyzed for their ability to transduce DC at various MOI (50, 100, 200 and 400) *in vitro* and the expression of β-gal protein was measured. DC transduced with HD-AdV/β-gal expressed from 150- to 1200-fold higher levels of β-gal protein than did DC transduced with FG-AdV/β-gal at the same MOI (Figure 4). Similarly, when DC were transduced with the same BFU (50, 100 and 200) of HD-AdV/β-gal or FG-AdV/β-gal, reproducibly higher steady-state levels of β-gal mRNA were demonstrated in HD-AdV-transduced cells by semiquantitative RT-PCR (Figure 5a). The relative ratio of mRNA encoding for β-gal and GAPDH (β-gal/GAPDH ratio) were quantitated by densitometry at the

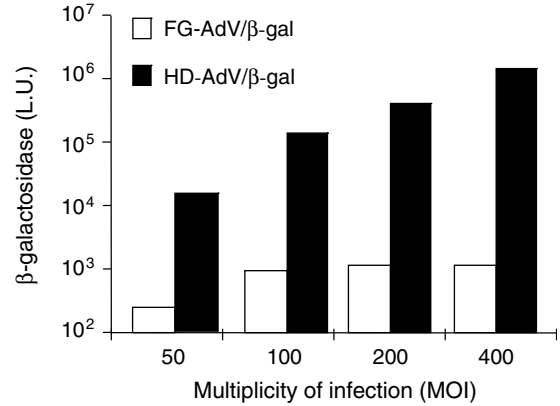


Figure 4 Dendritic cells transduced *in vitro* with HD-AdV/β-gal express 150–1200 higher levels of β-gal enzyme activity compared to DC transduced with FG-AdV/β-gal. Mouse bone marrow-derived DC were transduced with vectors at multiplicity of infections (MOI) ranging from 50 to 400 and 48 h later cell lysates examined for the expression of β-gal by enzyme-based luminescence assay. Results expressed as the average number of relative light units (LU) from triplicate determinations. Representative data from one of eight experiments.

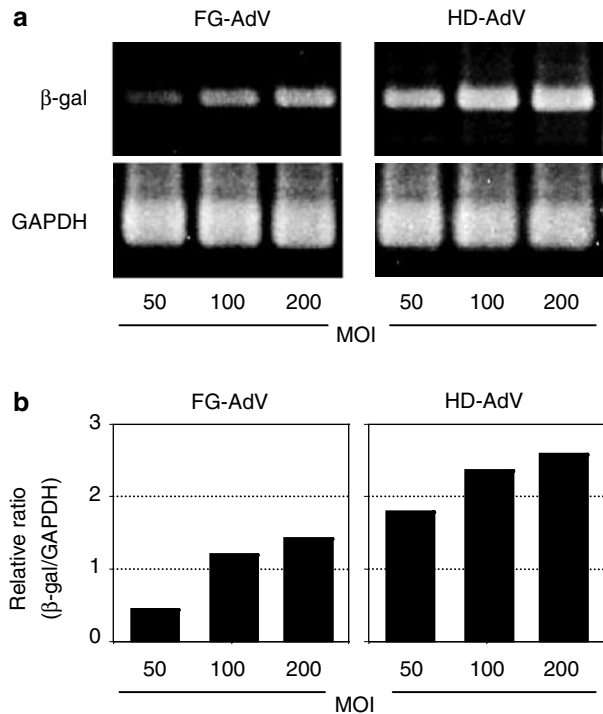


Figure 5 Enhanced β-gal expression in DC transduced with HD-AdV/β-gal results from greater transgene expression as determined by β-gal-specific RT-PCR (a). Relative ratio of transgene-β-gal and GAPDH was used as a measure for densitometric transgene expression (b). Mouse bone marrow-derived DC were transduced with either HD-AdV/β-gal or FG-AdV/β-gal at an MOI of 50, 100 and 200 and total mRNA extracted after 48 h for determination of gene expression by RT-PCR (35 cycles). Relative ratios (β-gal/GAPDH) at each of the MOI were calculated after transduction by dividing the β-gal value by GAPDH value observed after transduction of DC with FG-AdV or HD-AdV at different MOI (50, 100 and 200). Representative data are from one of six experiments.

different MOI (50, 100 and 200) (Figure 5b). The β-gal/GAPDH ratio obtained from DC transduced with a 50 MOI of HD-AdV/β-gal was higher than that produced by a 200 MOI of FG-AdV/β-gal (Figure 5b). These results

are similar to findings with human DC, where DC transduced with either HD-AdV/ β -gal or FG-AdV/ β -gal expressed similar levels of LacZ gene by PCR (suggesting identical viral transduction efficiencies), but HD-AdV/ β -gal produced higher levels of β -gal transgene by RT-PCR (indicating greater transgene expression).⁷

DC transduced with HD-AdV/ β -gal induce β -gal-specific CTL with a limited anti-AdV response

Consistent with the effects observed following direct viral injection, the CTL response generated against β -gal was significantly higher in mice immunized with HD-AdV/ β -gal transduced DC than in mice vaccinated with FG-AdV/ β -gal transduced DC ($P < 0.01$; Figure 6a). However, the magnitude of the CTL response was weaker than that resulting from direct injection of HD-AdV/ β -gal, suggesting that further optimization of the cell-based vaccine might be required. In addition, regardless of the vector used, vaccination with transduced DC failed to stimulate anti-AdV antibody responses (Figure 6b), suggesting that it might be possible to stimulate transgene-specific CTL with little anti-AdV response when using transduced DC as the immunogen.

LPS enhanced the expression of costimulatory molecules on AdV-transduced DC

It has been reported that DC maturation enhances the expression of costimulatory molecules and increases their capacity to stimulate antigen-specific T cells.¹¹ Prior to using this approach to boost the vaccine response, DC

were transduced with HD-AdV/ β -gal or FG-AdV/ β -gal at MOI of 50–200, cultured for 48 h in either control medium or medium containing 100 ng/ml LPS, and evaluated for their costimulatory molecule expression (CD40, CD80, and CD86). Transduction of DC with HD-AdV/ β -gal or FG-AdV/ β -gal induced negligible changes in costimulatory expression (Table 1). However, when DC transduced by either vector were matured in the presence of LPS, the mean fluorescence intensity (MFI) for CD40, CD80 and CD86 increased considerably (Table 1). There was no impact of LPS on the expression of β -gal (not shown). These results suggest that transduction of DC with a low MOI of either HD-AdV/ β -gal or FG-AdV/ β -gal (MOI 50–100) fails to activate or mature DC. However, these transduced DC can be effectively matured by short-term exposure to LPS.

Immunization using LPS-matured and transduced DC or DC transduced with a higher viral load, increased the stimulation of anti- β -gal CTL responses

Two approaches for enhancing transgene-specific CTL were examined. First, DC were loaded with a higher viral titer (MOI 200) as a mechanism for increasing antigen expression. Alternatively, DC transduced with an MOI of 50 were matured with LPS in order to enhance the efficiency of antigen presentation without increasing viral loading. When compared to mice immunized with HD-AdV/ β -gal-transduced DC using an MOI of 50, both of these approaches resulted in a two to three fold increase in β -gal-specific CTL (Figure 7a). These responses to AdV-transduced DC were now equivalent to those observed following direct AdV injection (Figure 1).

Despite potent CTL responses, AdV-transduced and LPS-stimulated DC produced only weak anti-AdV and anti- β -gal antibody responses

DC transduced with HD-AdV/ β -gal (50 MOI) and matured in presence of LPS were also evaluated for their ability to induce antibody against viral capsid and transgene antigens (Figure 7b). In contrast to direct immunization with HD-AdV, which stimulated potent antibody responses against both AdV and β -gal (Figure 2), there were no detectable antibody responses to either of these antigens when evaluated at the level of total Ig (not shown). However, evaluation of the IgG2a isotype response revealed weak but significant antibody

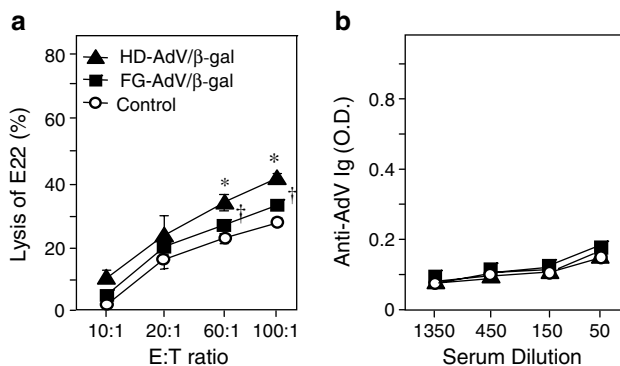


Figure 6 Immunization with DC transduced *in vitro* with HD-AdV/ β -gal stimulates a more potent transgene-specific CTL response than immunization with DC transduced with FG-AdV/ β -gal, with little evidence for the induction of antiadenovirus specific Ig antibodies. Mice were immunized twice at 7 days interval with 5×10^5 control DC or DC transduced with a 50 MOI of either HD-AdV/ β -gal or FG-AdV/ β -gal. (a) Splenocytes were collected 7 days after the last immunization and the CTL response against E-22 tumor cells determined as described in Figure 1. Representative data are from one of four experiments. * $P < 0.01$ comparing mice immunized with DC transduced with HD-AdV/ β -gal to mice immunized with either control DC or DC transduced with FG-AdV/ β -gal. † $P < 0.05$ comparing mice immunized with DC transduced with FG-AdV/ β -gal to mice immunized with control DC. (b) Serum was collected 7 days after the last immunization and the presence of adenovirus-specific antibody (Ig) detected by ELISA using irradiated FG-AdV/RR5 as the immobilized antigen. Data represent the mean \pm s.e. optical density (OD) measured for serial dilutions of sera pooled from two animals per group, assayed in triplicate. No significant increase in antiadenovirus-specific antibody (Ig) was detected in mice immunized with DC transduced with either FG-AdV/ β -gal or HD-AdV/ β -gal. Representative data are from one of four experiments.

Table 1 Exposure to LPS upregulate the expression of activation markers on DC

DC group ^a	Activation markers (MFI)		
	CD40	CD80	CD86
DC alone	50 ^b	90	130
+FG-AdV/ β gal	55	118	165
+HD-AdV/ β gal	69	125	177
+FG-AdV/ β gal+LPS	125	174	201
+HD-AdV/ β gal+LPS	135	180	214

^aBM-derived DC were cultured alone or transduced with either FG-AdV/ β -gal or HD-AdV/ β -gal (MOI 50) and then cultured for 48 h in the presence or absence of 100 ng/ml LPS.

^bDC were identified by the presence of CD11c and the expression of each activation marker reported as the mean fluorescence intensity of 5000 events as determined by FACS analysis.

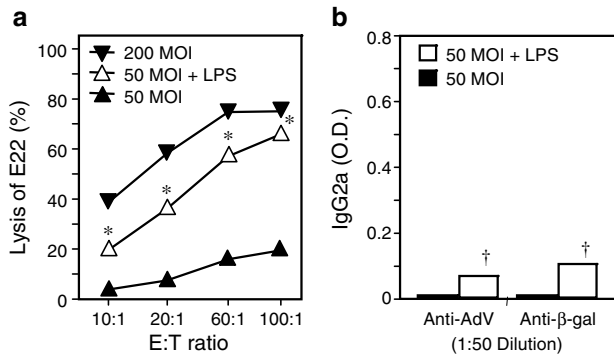


Figure 7 Effect of LPS activation of HD-AdV/ β -gal transduced DC on transgene-specific CTL and antibody response against AdV vector and transgene. (a) Transducing DC with a higher MOI of HD-AdV/ β -gal, or activating them with LPS, results in the induction of higher β -gal-specific CTL responses when used for vaccination. Mouse bone marrow-derived DC were transduced with HG-AdV/ β -gal at an MOI of either 50 or 200, or with an MOI of 50 followed by treatment with 100 ng/ml of LPS. Mice were immunized twice at 7 day interval with 5×10^5 DC and splenocytes collected 7 days later for determination of transgene-specific CTL as described in Figure 1. * $P < 0.01$ comparing mice immunized with DC transduced with HD-AdV/ β -gal at a MOI of 200 to mice immunized with either a MOI of 50 alone or treated with LPS. † $P < 0.01$ comparing mice immunized with DC transduced with a MOI of 50 in combination with LPS to DC transduced with a MOI of 50 alone. Representative data are from one of three experiments. * $P < 0.01$ comparing splenocytes from HD-AdV/ β -gal + LPS immunized mice to splenocytes from HD-AdV/ β -gal and FG-AdV/ β -gal (200 MOI) immunized mice. Representative data from one of four experiments. (b) Immunization with transduced and LPS-treated DC results in minimal but significant changes in IgG2a isotype specific antiadenoviral and anti- β -gal antibody response. Serum was collected 7 days following the last immunization and evaluated for IgG2a antibody responses to adenoviral capsid or β -gal protein by ELISA assay. Data represent the mean \pm s.e. optical density (OD) measured for a 1:50 dilution of sera pooled from two animals per group, assayed in triplicate. † $P < 0.01$ comparing sera from mice immunized with HD-AdV/ β -gal transduced and LPS-activated DC compared to sera from mice immunized with HD-AdV/ β -gal transduced DC only. Sera from control mice immunized with LPS treated untransduced DC showed OD values similar to sera from mice immunized with transduced DC (50 MOI).

response to both AdV capsid antigens and β -gal protein (Figure 7b). Thus, while immunization with transduced and LPS-matured DC induced high levels of β -gal specific CTL similar to that occurring following direct viral immunization, the antibody responses to both the vector and transgene were attenuated by this route.

Discussion

FG-AdV are rendered replication deficient by deletion of E1 genes (Δ E1) and exhibit several attractive properties for gene therapy including a large transgene carrying capacity, ease of construction and propagation, limited cytotoxicity, and efficient transduction of both resting and mitotic cells.^{12,13} Components of the adenoviral capsid can also act as immune adjuvants, further potentiating their use as gene-based vaccines.^{14,15} In animal models, immunization with a FG-AdV produces more effective antitumor immunity than other common approaches including immunization with peptides, proteins, or plasmid DNA.^{16,17} However, in contrast to animal models, antiviral immunity exists in the majority of patients^{18,7} and the combination of neutralizing antiviral antibodies and antiviral T cells can impair

transgene expression and the generation of transgene-specific CTL.^{2,19,20} Early work by Yang *et al.*²¹ suggested that *in vivo* responses to FG-AdV are directed against viral gene products expressed *de novo* following transduction. To address this obstacle, HD-AdV were designed that lack all viral-coding regions.^{4,6} When administered *in vivo*, HD-AdV express significantly higher levels of transgene for significantly longer periods of time than FG-AdV.⁹ Intravenous administration of a HD-AdV expressing leptin as the transgene resulted in 10-fold higher leptin levels compared to treatment with FG-AdV.²² While administration of the FG-AdV was associated with hepatic injury, administration of the HD-AdV was not. Further, mice treated with the FG-AdV demonstrated significant loss of transgene cDNA by 2 weeks, while expression persisted with little change for 8 weeks following administration of HD-AdV. Similarly, Zou *et al.*²³ injected either a FG-AdV or a HD-AdV directly into brain tissue *in vivo*. Transgene expression by the FG-AdV dropped to low levels by day 33, while significant expression persisted for at least 2 months in animals treated with the same dose of HD-AdV. Histology also identified tissue injury and localized infiltration by macrophages and lymphocytes in animals that received the FG-AdV, but not in response to HD-AdV. Collectively, these changes suggest that antiviral immunity develops against the FG-AdV, but is avoided by use of a HD-AdV. However, the capacity for HD-AdV to evade immune detection has recently been called into question. Parks *et al.*²⁴ demonstrated that the heightened transgene expression associated with HD-AdV results from the inclusion of transcriptional enhancers within the vector backbone, not a difference in host response. When Maione *et al.*⁸ administered titrating doses of HD-AdV and FG-AdV to cohorts of mice, both vectors stimulated equivalent and dose-dependent levels of antiviral antibody. Similarly, we reported that human DC transduced with either FG-AdV or HD-AdV activated the same frequency of viral-specific T cells *in vitro*. The generation of antiviral T cells was dose-dependent and occurred even when viral transcription was blocked, suggesting that preformed components of the viral capsid were acting as the major source of antigens.⁷ Finally, O'Neal *et al.*²⁵ administered AdV to both immunodeficient and immunocompetent mice. Hepatotoxicity occurred only in response to the FG-AdV and was most pronounced in immunodeficient mice, implying that viral genes expressed *de novo* exert a toxic effect that may be unrelated to antigen-specific immunity. As such, HD-AdV may express high levels of transgene for extended periods of time when administered *in vivo*, but the extent to which it evades host detection remains to be clarified. As our studies focused on the early immune responses occurring within 7–14 days of immunization, directed primarily against capsid antigens, it is possible that we missed differences in delayed immunity against viral genes expressed *de novo* by the FG-AdV but not by the HD-AdV. Additional studies designed to investigate late viral expression and CTL responses against *de novo* viral antigens should be considered before drawing a conclusion in this respect.

In the current studies, we hypothesized that the heightened transgene expression resulting from a HD-AdV, regardless of the mechanism, would translate into a more potent stimulation of transgene specific immunity.

The capacity for HD-AdV to act as an immunizing vector *in vivo* was therefore evaluated using two different vaccine strategies. In the first, mice were directly immunized with either HD-AdV/ β -gal or FG-AdV/ β -gal and monitored for the induction of β -gal-specific CTL, β -gal-specific antibody, and anti-AdV antibody. Even with a single immunization, HD-AdV/ β -gal stimulated substantially higher transgene specific CTL responses than an equivalent dose of FG-AdV/ β -gal. This effect was boosted by a second vaccination and paralleled by the development of anti- β -gal antibodies of the IgG2a isotype, consistent with the induction of a strong Th1 response.²⁶ Inbred mice are naïve to adenoviral infection and differences in the primary immune response to these two vectors were not likely due to differences in the intensity of antiviral immunity. Consistent with this, we found that mice immunized with either vector developed nearly identical antiviral antibody responses. The capacity for HD-AdV to stimulate a potent Th1 response against β -gal was most likely due to its capacity for enhanced transgene expression. Other gene-based vaccine models have demonstrated a clear relationship between the level of gene expression and the induction of antigen-specific CTL.^{27,28}

The induction of viral-specific antibodies was closely monitored in order to gain further insight into the nature of the antiviral response. A single immunization with either type of AdV produced significant antibody responses that were identical in magnitude (data not shown). When immunized a second time, there was a further increase in anti-AdV titer in both treatment groups. While the response to FG-AdV was slightly higher, the magnitude of this difference was small and likely unimportant. The anti-AdV antibody responses belonged to both the IgG1 and IgG2a isotypes as has been reported earlier.⁶⁰ It appears that the viral capsid used to package HD-AdV still induces potent humoral immunity and that neutralizing antiviral antibody is likely to remain an obstacle when employing HD-AdV for vaccine purposes.

Antigen-presenting cells are important in the response to viral infections and direct transduction of DC *in vivo* appears to be the major pathway responsible for induction of cellular immunity.^{27–29} As such, we next examined the capacity for FG-AdV and HD-AdV to transduce murine DC *in vitro* and for these transduced cells to stimulate transgene-specific immune responses *in vivo*. Similar to our results with human monocyte-derived DC,⁷ we found a significantly higher expression of steady-state mRNA encoding for β -gal, and a 150- to 1200-fold higher expression of β -gal protein, in mouse DC transduced with HD-AdV/ β -gal. This heightened expression of β -gal protein in DC transduced by a HD-AdV may be due to replacement of inhibitory viral-coding regions with specific stuffer DNA sequences that enhance expression of, or prolong the retention and/or stability of, transgene mRNA as previously described.²⁴ The basic mechanisms responsible for modest increases in steady-state mRNA, but dramatic increases in expression of transgene protein by HD-AdV-transduced DC, warrants further investigation. Regardless of the exact mechanism, these findings led us to hypothesize that loading of DC with a HD-AdV *ex vivo*, followed by administration as a cell-based vaccine, might promote effective transgene responses while limiting viral ex-

posure. Recombinant AdV has been used extensively to transfer genes into murine and human DC and to stimulate antitumor immunity in animal models.^{12,13,30–32} More importantly, the strength of the CTL response has been shown to depend upon the magnitude of antigen expression.^{30–32} Consistent with this, we found that DC transduced with HD-AdV/ β -gal stimulated superior CTL responses *in vivo* when compared to DC transduced with the same MOI of FG-AdV/ β -gal. Equally important, AdV-transduced DC induced only minor anti-AdV antibody responses when compared to direct vaccination with the AdV. This cell-based approach has been suggested as an effective technique for avoiding antiviral responses that occur with direct and repeated delivery of the virus itself.^{32,33} There may be several reasons for this. First, as no free virus is administered, transduced cells escape the effects of pre-existing neutralizing antibodies directed against the viral capsid. Second, even though transduced DC are loaded with viral antigens, and can stimulate antiviral immunity, the total viral burden delivered by 5×10^5 transduced DC is considerably less than that delivered by a direct injection of 10^8 BFU of virus. Finally, the route of antigen processing may alter the relative balance between cellular and humoral responses. The endogenous processing of antigens directly within transduced DC favors loading into MHC class I and therefore effector T-cell responses. In contrast, the exogenous delivery of antigens by bystander cells that are transduced *in vivo* may result in cross-priming of DC and a more balanced processing into both MHC class I and II.^{26,34,35} When Mercier *et al*²⁶ compared the direct injection of FG-AdV to vaccination with AdV-transduced DC, they noted relatively similar cell-mediated responses after 11 days, but antibody titers were 100-fold higher in the animals that were vaccinated with the FG-AdV *in vivo*.

Despite the potential advantages inherent in using transduced DC as a vaccine, the magnitude of the antigen-specific CTL response was relatively low when compared to direct viral immunization. Efforts were therefore made to optimize the DC-based vaccination. In addition to increasing the level of antigen expression by increasing the MOI, the DC maturation state may play an important role in determining the magnitude of a T-cell response.³⁶ DC can be matured by various stimuli including microorganisms, LPS, CPG motifs, or cytokines.^{37,38} Activation in this manner leads to upregulation of MHC and costimulatory molecules such as CD80 and CD86.^{39,40} Activation also alters expression of chemokine receptors resulting in better trafficking to secondary lymphoid organs, increases IL-12, and skews T-cell responses toward more effective Th1 profiles.^{41–43} We therefore explored the effects of DC maturation by treating cells with LPS. It has been reported that DC are matured by AdV transduction itself, but this effect is relatively weak and dependent upon the viral load.^{41,44,45} When we analyzed the activation state of DC transduced with HD-AdV/ β -gal and FG-AdV/ β -gal (MOI 50–200 BFU/cell), there were negligible changes in marker expression (Table 1). In contrast, exposure to LPS significantly increased expression of CD80, CD86 and CD40. It has also been reported that mature DC exhibit higher transgene expression. This effect might result from higher viral uptake⁴⁶ or NF- κ B-dependent signaling.^{47,48} In our hands, there was no difference in

the expression of β -gal protein when transduced cells were treated with LPS (not shown). This may relate to low MOI (50–200) of virus used for transduction of DC or differences in timing or culture conditions as compared to prior studies. *In vitro* modifications were evaluated for their impact on *in vivo* outcomes by immunizing mice with DC that were transduced at an MOI of 50, at a higher MOI of 200, or at an MOI of 50 in combination with LPS. There was a significant and fairly dramatic increase in the production of β -gal-specific CTL activity in response to both types of manipulation. The CTL response following LPS was almost the same as that occurring in response to transducing DC with four times the amount of HD-AdV. DC maturation may be an effective mechanism for increasing antigen-specific vaccine responses, while limiting viral exposure. As further evidence of this, we examined the impact of LPS activation on the generation of anti-AdV *versus* anti- β -gal antibody. The use of LPS-treated DC for immunization resulted in low but detectable IgG2a antibody responses against both β -gal and AdV. These results confirm that activation of DC has a relatively minor impact on the stimulation of anti-AdV antibody responses, while promoting a strong transgene-specific response that is primarily cell-mediated and consistent with a Th1 profile.

In conclusion, our results with HD-AdV suggest a role for this new generation vector in gene-based vaccine strategies. HD-AdV induces dramatically higher transgene expression for the same amount of viral exposure. This heightened expression translates into better antigen presentation and more potent CTL responses regardless of whether the vector is administered directly *in vivo* or in the form of *ex vivo* transduced DC. Vaccination with transduced DC requires less virus and stimulates only limited antiviral antibody responses from the host. In addition, transduced DC are not neutralized by pre-existing antiviral antibody. As such, there may be distinct advantages to cell-based gene vaccination. Other mechanisms for boosting transgene expression and DC presentation might be applied to HD-AdV. DC maturation offers one such approach that lowers the required viral titer another three- to four-fold. Alternative capsids with a higher efficiency for entering DC, such as the AdV35 serotype, RGD motifs, or redirection to CD40 receptors, are another alternative.^{49–51} However, if these approaches load more virus into DC they may also increase the stimulation of antiviral responses. The balance between viral load and transgene expression is likely a crucial factor. Higher efficiency promoters or enhancing elements, such as those derived from the E4 region of adenovirus, have been reported to increase transgene expression and might also be combined into the design of HD-AdV.^{52,53} Further development of these approaches might significantly enhance the efficacy and safety of gene-based vaccination.

Materials and methods

Mice and cell lines

Male C57BL/6 mice (H-2^b), 8–12 weeks old, were purchased from Charles River Laboratories (Wilmington, MA, USA) and were housed at UCLA. Procedures involving mice were approved by the UCLA Animal

Research Committee. EL-4, a syngeneic murine thymoma cell line (ATCC, Manassas, VA, USA), and a retroviral-transduced clone of EL-4 expressing the LacZ gene, E22 (kindly provided by Dr NP Restifo, National Cancer Institute, Bethesda, MD, USA), were used as target cells for determining both antigen nonspecific and β -gal-specific CTL activity, respectively.^{11,54}

Cytokines

Recombinant mouse GM-CSF (rGM-CSF, specific activity $\geq 5 \times 10^6$ U/mg) and IL-4 (rIL-4, specific activity $\geq 1 \times 10^7$ U/mg) were acquired from Peprotech (Rocky Hill, NJ, USA).

Adenoviral vectors

Three different AdV were utilized: FG-AdV/ β -gal, a first-generation AdV ($\Delta E1/\Delta E3$, serotype 5) containing the LacZ transgene under a CMV enhancer/promotor (Qbiogene, Carlsbad, CA, USA); AdRR5, another first-generation AdV constructed on the same background, but containing only the CMV promoter without a transgene insert (kindly provided by Drs L Butterfield and J Economou, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA);¹⁷ and HD-AdV/ β -gal, a helper-dependent AdV containing the LacZ transgene under the same CMV enhancer/promoter.^{55,56} HD-AdV/ β -gal was constructed using an E-1 deleted helper virus with lox sites flanking the packaging signals (AdLC8Luc; Merck, Whitehouse Station, NJ, USA) and a 293 cell line expressing Cre recombinase (293-Cre4 cells; Merck) as previously described.⁶ The titers for FG-AdV/ β -gal and HD-AdV/ β -gal were determined to be 1.1×10^{11} and 7.3×10^9 blue-forming units (BFU)/ml, respectively, using 293 according to an established protocol.⁶ Contamination by helper virus in the HD-AdV/ β -gal stock was determined to be $< 0.01\%$ by standard plaque assay. Stock preparations of FG-AdV/ β -gal and HD-AdV/ β -gal were also evaluated by spectrophotometry at 260 nm to determine viral particle number by the following equation: particle number = $(OD_{260}) \times (1.1 \times 10^{12})$. The particle:BFU ratio for FG-AdV/ β -gal and HD-AdV/ β -gal were calculated to be 13.2:1 and 12.6:1, respectively. These carefully titrated FG-AdV/ β -gal and HD-AdV/ β -gal were used to immunize mice and infect DC at different multiplicity of infection (MOI).

Preparation and transduction of bone marrow-derived murine DC

DC were generated from murine bone marrow (BM) by the method of Inaba *et al*⁵⁷ Briefly, BM cells were harvested and depleted of NK, B, T and granulocytes by antibody (Pharmingen, San Diego, CA, USA) and complement (Sigma, St Louis, MO, USA), were cultured overnight in (RPMI 1640 (Irvine Scientific, Santa Ana, CA, USA) with 10% heat-inactivated fetal bovine serum (FBS; Omega Scientific, Tarzana, CA, USA), HEPES (Sigma), 2-mercaptoethanol (Sigma) and Penicillin–Streptomycin (Sigma)). The next day, adherent cells were removed and 20 ng/ml each of rGM-CSF and rIL4 were added. Cell cultures were incubated for 7 days with a media change every 3 days.

BM-derived DC were transduced with AdV as described previously by Roth *et al*.⁷ Briefly, 5×10^5 DC were exposed to a suspension of AdV at different MOI

in 100 μ l complete medium. After 2 h at 37°C, 800 μ l of complete media containing 20 ng/ml each of GM-CSF and IL-4 was added and cultures incubated for another 48 h before analysis or injection into mice. The β -gal expression was evaluated by a luminescence-based enzyme assay (Clontech, Palo Alto, CA, USA) using cell lysates as described earlier, with results reported in relative light units (RLU).⁷

RT-PCR

Total cellular RNA was isolated using RNeasy Mini Kits (Qiagen Inc., Valencia, CA, USA). For RT-PCR, 1 μ g of total RNA was used to synthesize cDNA with cDNA Cycle Kit (Invitrogen: Carlsbad, CA, USA) and 1 μ l of the RT-product was used as template to perform PCR. For PCR, 5 ng DNA was used as template and amplified with 35 cycles of 45 s at 92°C, 45 s at 58°C, and 1 min at 72°C. β -Gal was detected by amplifying the fragment from 2884 to 3366 bp as the primer sequence and GAPDH was detected by amplifying the fragment from 667 to 981 bp. RT-PCR products were analyzed by 1.2% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. Signal intensities were quantified by densitometry using NIH Image 1.62 software to obtain β -gal:GAPDH ratios.

AdV immunization protocols

Two approaches to AdV-based immunization were investigated, including direct injection with AdV *in vivo* and vaccination with DC that had been transduced with AdV *in vitro*. For the *in vivo* approach, 1×10^8 BFU of either HD-AdV/ β -gal or FG-AdV/ β -gal were delivered by the intraperitoneal route (i.p.) either once (day 0) or twice (days 0 and 7). To determine the effect of DC-mediated vaccination, mice were immunized with either control DC or DC that had been transduced 48 h earlier with 50 MOI of either HD-AdV/ β -gal or FG-AdV/ β -gal. In some experiments, the MOI was increased to 200 or the DC were stimulated to mature with 100 ng/ml LPS added to the cultures following AdV transduction. 5×10^5 DC were suspended in 100 μ l PBS and injected subcutaneously (s.c.) on days 0 and 7. Sera were collected 7 days after the last immunization and assayed for the development of antibody responses to either AdV capsid antigens or β -gal protein. Spleen cells were harvested at the same time and used to evaluate CTL responses against β -gal.

CTL assay

CTL assays were performed as described previously.⁵⁸ Briefly, splenocytes were pooled from two mice per group and restimulated *in vitro* with irradiated E22 (EL-4 cells expressing β -gal protein) cells cultured at a 25:1 (splenocyte:E22) ratio for 5 days in the presence of 10 U/ml of IL-2. Cytotoxicity was assessed in a standard 4-h chromium release assay with lysis of chromium-labeled E22 cells (β -gal-specific) and lysis of parental EL-4 cells (nonspecific) tumor killing. Maximum and spontaneous release were determined for each target and used to calculate % lysis.

Anti-AdV and anti- β -gal antibody assays

Anti-AdV antibodies were determined by ELISA as described previously.^{59,60} Microtiter plates (96-well) (Corning Inc., Fountain Valley, CA, USA) were coated

overnight at 4°C with 50 μ l of coating buffer (200 mM NaHCO₃, 81 mM Na₂CO₃, pH 9.5) containing 1×10^{11} particles/ml of UV-irradiated AdRR5. Wells were then washed 4 times with PBS/0.05% Tween-20 and blocked in PBS/3% BSA for 3 h at room temperature. Pooled serum samples from two mice per group were diluted 1:50, 1:150, 1:450 and 1:1350 with PBS, added to antigen-coated wells, and incubated overnight at 4°C. Wells were then washed 4 times with PBS/0.05% Tween-20 and incubated with biotin-conjugated goat anti-mouse polyclonal antibody reactive to either total mouse Ig (1:5000 dilution, BD Biosciences Pharmingen, San Diego, CA, USA) or specific to mouse IgG1 or IgG2a (1:5000 dilution, Caltag Laboratories, Burlingame, CA, USA), for 3 h at room temperature. For detection, plates were washed and incubated with a 1:50 000 dilution of alkaline phosphatase-conjugated avidin (Sigma) for 2 h at room temperature. After four washes, *p*-nitrophenyl phosphate diethanolamine buffer was added to each well and optical densities were read at 405 nm on a microplate reader (Dynatech Laboratories, Chantilly, VA, USA). The presence of anti- β -gal antibody was determined by an identical ELISA approach replacing a solution of 500 ng/ml purified soluble β -gal (Sigma) for the suspension of adenovirus in the coating buffer. Preimmune sera were used to determine background levels and these values were subtracted from the experimental values. The results are presented as \pm s.e. The differences are analyzed by the Student's *t*-test.

DC phenotyping

BM-derived DC were transduced with HD-AdV/ β -gal and then either cultured in complete media containing 20 ng/ml GM-CSF/IL-4 or matured in the presence of 100 ng/ml LPS for 48 h. The resulting DC were analyzed by fluorescence-activated cell sorter (FACS) for the expression of several DC markers using PE-conjugated anti-CD11c (Caltag Laboratories) to identify DC and biotin-labeled mAb-directed against CD80, CD86 or CD40 (Caltag Laboratories) in combination with a secondary streptavidin-APC (Caltag Laboratories). The acquisition of up to 10^5 events was performed using a FACStar cytometer (Becton Dickinson, San Jose, CA, USA) and results analyzed using CellQuest software (Becton Dickinson).

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