CD40 Ligand-Dependent Activation of Cytotoxic T Lymphocytes by Adeno-Associated Virus Vectors In Vivo: Role of Immature Dendritic Cells

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Recombinant adeno-associated virus type 2 (rAAV) is being explored as a vector for gene therapy because of its broad host range, good safety profile, and persistent transgene expression in vivo. However, accumulating evidence indicates that administration of AAV vector may initiate a detectable cellular and humoral immune response to its transduced neo-antigen in vivo. To elucidate the cellular basis of the AAV-mediated immune response, C57BL/6 mouse bone marrow-derived immature and mature dendritic cells (DCs) were infected with AAV encoding β-galactosidase (AAV-lacZ) and adoptively transferred into mice that had received an intramuscular injection of AAV-lacZ 10 days earlier. Unexpectedly, C57BL/6 mice but not CD40 ligand-deficient (CD40L−/−) mice adoptively transferred with AAV-lacZ-infected immature DCs developed a β-galactosidase-specific cytotoxic T-lymphocyte (CTL) response that markedly diminished AAV-lacZ-transduced gene expression in muscle fibers. In contrast, adoptive transfer of AAV-lacZ-infected mature DCs failed to elicit a similar CTL response in vivo. Our findings indicate, for the first time, that immature DCs may be able to elicit a CD40L-dependent T-cell immunity to markedly diminish AAV-lacZ transduced gene expression in vivo when a sufficient number of DCs capturing rAAV vector and/or its transduced gene products is recruited.

The ability of a vector and its transduced gene to persist is one of the important goals for gene therapy. Vectors that are highly efficient at delivering genes in a tissue-specific manner, without inducing strong cellular immune response, are of great interest for gene therapy. Adeno-associated virus type 2 (AAV) has become an attractive tool for gene therapy due to its broad host range, excellent safety profile, and durable transgene expression in infected hosts (7, 12, 13, 19, 20, 37). Intramuscular injection of AAV vectors does not stimulate a cellular immune response to highly expressed neoantigenic transgene products in immunocompetent mice (12, 19, 37), whereas other vector systems expressing the identical transgene, such as adenovirus (40) and naked DNA (36), do. These studies illustrate the role of the AAV vector in modulating (or avoiding) immune responses to the transgene through an unknown mechanism.

Accumulating evidence indicates that the lack of destructive cellular immunity by AAV vectors may depend on the transgene involved and the route of administration (8, 21, 22). In contrast to the long-term expression of β-galactosidase (βGal) in muscles of mice, it has been found that inoculation of AAV encoding βGal (AAV-βGal) into the brains of BALB/c mice induced βGal expression during the first 2 months and that this expression decreased gradually by 4 months. Moreover, repeated administration of AAV-lacZ vector into the brains of mice resulted in a loss of βGal expression in the original injection sites in 2 of 6 animals (21). Intramuscular injection of AAV vector encoding herpes simplex virus type 2 glycoproteins B and D (AAV-gB and AAV-gD) induces both humoral and cellular immune responses to these antigens (22). More recently, it has been demonstrated that C57BL/6 mice injected via the intraperitoneal, intravenous, or subcutaneous route with AAV encoding ovalbumin (AAV-ova) developed potent ovalbumin-specific cytotoxic T-lymphocyte (CTL) response as well as anti-ovalbumin antibodies. In contrast, mice intramuscularly injected with AAV-ova developed a humoral response to the virus and the transgene product but minimal ovalbumin-specific CTL (8). All these data challenge the claims that AAV vectors are nonimmunogenic. However, the cellular basis of AAV vector-mediated immune response in vivo remains to be elucidated.

Our previous study demonstrated that adoptive transfer of dendritic cells (DCs) infected with adenovirus (Ad)-lacZ vector leads to immune-mediated elimination of AAV-lacZ-transduced gene expression in muscle fibers in immune competent mice (17). This study underscored the critical role of vector-transduced DCs in initiating cellular immune responses. DCs are antigen-presenting cells that specialize in initiating T-cell immunity, including CTLs that kill virus-infected targets. DCs normally reside in tissues in an immature form with the capacity to capture antigen. After antigen capture, and in response to inflammatory stimuli, immature DCs switch to a T-cell-stimulatory mode to initiate cellular immunity (2, 4, 11, 23, 26, 28, 31, 34). However, it remains to be determined if immature DCs take up AAV vector and play a role in modulating the immune response to the AAV-carried transduced gene in vivo.

To investigate the mechanism of the AAV vector-mediated cellular immune response, purified immature and mature DCs that were generated from murine bone marrow (BM) hematopoietic progenitor cells (HPCs) (41, 42) were infected with either AAV-lacZ or Ad-lacZ and adoptively transferred into C57BL/6 and CD40 ligand-deficient (CD40L−/−) mice. We show that immature DCs can take up AAV-lacZ vector and initiate a CD40L-dependent T-cell immunity in vivo to the AAV-transduced gene in muscle fibers.
TABLE 1. Overview of the experimental strategy

<table>
<thead>
<tr>
<th>Group</th>
<th>Vector</th>
<th>Adoptive transfer</th>
<th>Abbreviation</th>
<th>No. of recipient mice</th>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>C57BL/6</td>
</tr>
<tr>
<td>1</td>
<td>AAV-lacZ</td>
<td>AAV-lacZ PBS</td>
<td>AAV-lacZ PBS</td>
<td>12</td>
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<tr>
<td>2</td>
<td>AAV-lacZ</td>
<td>Control mature DCs</td>
<td>Mature DCs</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>AAV-lacZ</td>
<td>Ad-lacZ-infected immature DCs</td>
<td>Ad-lacZ immature DCs</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>AAV-lacZ</td>
<td>AAV-lacZ-infected immature DCs</td>
<td>AAV-lacZ mature DCs</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>AAV-lacZ</td>
<td>AAV-lacZ-infected mature DCs</td>
<td>AAV-GFP immature DCs</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>AAV-lacZ</td>
<td>AAV-GFP-infected DCs</td>
<td>Immature DCs</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>AAV-lacZ</td>
<td>Control immature DCs</td>
<td></td>
<td>ND</td>
</tr>
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* Wild-type C57BL/6 and CD40L−/− mice were injected intramuscularly (i.m) with AAV-lacZ in the left tibialis anterior on day 1. Immature and mature DCs were generated from C57BL/6 mouse BM and infected with various DNA viral vectors as described in Materials and Methods. PBS, control DCs, and various DC populations were not infected by any viral vector. The left tibialis anterior was harvested 28 days later, after adoptive transfer of various DCs, cryosectioned, and stained for lacZ expression. The instrument compensation was set in each experiment using a negative control. (1011 genomes/mouse) unless otherwise indicated. In two-color analyses, the cultured immature DCs were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD86, anti-CD40, anti-Ia, and anti-major histocompatibility complex class I and phosphotyrosin (PE)-conjugated anti-CD11c. In some experiments, the cultured cells were stained with PE-conjugated anti-CD86 and anti-Ia and FITC-conjugated anti-CD11c for intracellular staining to determine lacZ expression, or were fixed and permeabilized using the Cytofix/Cytoperm Plus kit (Pharmingen) and then subjected to biotinylated mouse anti-betaGal and FITC-conjugated streptavidin staining. The instrument compensation was set in each experiment using single- and/or two-color stained samples.

T-cell assays. Lymphocytes were isolated from the spleens and regional lymph node of mice 28 days after adoptive transfer of various DCs and prepared for T-cell assays. CTL assays were performed as previously described (12), by restimulating the single-cell suspension with betaGal (20 μg/ml; Sigma) for 5 days at 5 × 10^5 cells/ml. These cells were assayed on MC57 target cells at different effector-to-target-cell ratios (starting at 6.25:1) in a 6-h 51Cr-release assay. As target cells, MC57 cells were infected with Ad-lacZ (100 genomes/cell) for 16 h or a betaGal-expressing cell line (established by transducing MC57 cells with pLacZ retrovirus) was used (17). For cytokine enzyme-linked immunosorbent assays (ELISA), the lymphocytes (2 × 10^6 cells/ml) were cultured in the presence of interleukin-2 (IL-2) (50 U/ml; R&D Inc.) and restimulated with betaGal (20 μg/ml) for 72 h. The supernatants were harvested and analyzed for the secretion of gamma interferon (IFN-γ) and IL-10 by using an ELISA kit as recommended by the manufacturer (Pharmingen).

RESULTS

Interaction of AAV-lacZ vector ex vivo with murine BM-derived DCs. Culture of murine BM HPCs with GM-CSF, SCF, and TNF-α generated immature DCs with monocyte-like morphology by day 6 (Fig. 1A). These highly purified immature DCs, which were magnetically sorted using anti-CD11c antibody-conjugated microbeads, could differentiate into mature DCs, characterized by typical DC morphology (Fig. 1A) and increased expression of Ia, CD86, and CD40 (Fig. 1B), in...
response to GM-CSF plus TNF-α. These purified immature DCs were separately infected with Ad-lacZ (2 × 10^3 genomes/cell), AAV-lacZ (2 × 10^4 genomes/cell), or PBS (mock infection) at 37°C for 2 h and recultured in the presence of GM-CSF or GM-CSF plus TNF-α for an additional 3 days. Infection of immature DCs with Ad-lacZ moderately enhanced the expression of Ia, CD86, and CD40 antigens in the presence of either GM-CSF (Fig. 1C, column 1) or GM-CSF plus

FIG. 1. Interaction of AAV-lacZ with BM-derived immature DCs. (A) Immature DCs were generated from BM HPCs stimulated with GM-CSF, SCF, Flt-3L, plus TNF-α on day 6 and purified by magnetic-cell sorting using anti-CD11c-conjugated microbeads. CD11c^+ immature DCs were induced to differentiate into mature DCs with GM-CSF plus TNF-α for an additional 3 days. Giemsa staining shows the typical morphology of immature and mature DCs. Magnification, ×304. (B to D) These highly purified immature DCs and their mature counterparts were stained with FITC-conjugated anti-CD40, anti-CD86, and anti-Ia and subjected to flow cytometry analyses (B). CD11c^+ immature DCs were suspended in 400 µl of serum-free IMDM, infected with Ad-lacZ and AAV-lacZ for 2 h at an infectious activity of 2 × 10^3 and 2 × 10^4 genomes/cell, respectively, and recultured in the presence of GM-CSF or GM-CSF plus TNF-α for an additional 3 days. The cells were stained with PE-conjugated anti-Ia and anti-CD86 and FITC-conjugated anti-CD40 and subjected to flow cytometry analyses (C). Immature and mature DCs were infected separately with Ad-lacZ, AAV-lacZ, or AAV-lacZ plus wild-type Ad (100 genomes/cell), as indicated in panel C, cultured in the presence of GM-CSF plus TNF-α for 3 days, and subjected to intracellular staining with anti-βGal MAb and flow cytometry analyses (D). The x-axes of the histograms in panels B to D show a log scale of the fluorescence intensity of the tested antigens, and the y axes show a linear scale of the number of cells with a given fluorescence intensity. (E) The infected immature DCs were cytocentrifuged for X-Gal histochemistry staining. Magnification, ×122. The blue color indicates βGal-positive cells.
Adoptive transfer of AAV-lacZ-infected mature DCs could not diminish AAV-lacZ-transduced muscle fibers, whereas AAV-lacZ-infected immature DCs did so efficiently (Fig. 2B and C). Consequently, AAV-lacZ-infected mature DCs could not induce a significant CTL response to βGal antigen (Fig. 3B). These results demonstrate that the differences between immature and mature DCs in initiating an immune response to the AAV-transduced gene may result from the distinct transcription efficiency and differentiation states. It is possible that the susceptibility to AAV-lacZ and the potent capacity of antigen uptake and processing may enable immature DCs to initiate an immune response to diminish AAV-lacZ-transduced gene expression.

Adoptive transfer of AAV-lacZ-infected DCs initiates CD40L-dependent T-cell immunity. Lymphocytes were isolated from the spleens and regional lymph node of the recipient animals, as shown in Table 1, and cultured ex vivo to examine the cytokine release. Intramuscular injection of C57BL/6 mice with AAV-lacZ (Fig. 4A) but not with PBS (data not shown) could induce the production of IL-10, in agreement with our previous studies (12, 17). Adoptive transfer of AAV-lacZ-infected immature DCs further enhanced IL-10 secretion (Fig. 4A) and markedly induced an IFN-γ response in the recipients (Fig. 4B), implying activation of CD4⁺ T cells by AAV-lacZ-infected immature DCs. To further elucidate the role of CD4⁺ T cells, CD40L⁻/⁻ mice were used as recipients for the next set of experiments. Adoptive transfer of Ad-lacZ or AAV-lacZ-infected immature DCs failed to elicit a CTL response (Fig. 3C), resulting in persistent βGal expression in muscles of CD40L⁻/⁻ mice (Fig. 2A). Since DCs express CD40 but not CD40L molecules (4, 5), adoptive transfer of DCs derived from C57BL/6 mice into CD40L⁻/⁻ mice cannot supplement any additional CD40L signaling to activate T lymphocytes. These observations show that CD40L-CD40 interactions play a critical role in regulating immature DC-mediated immune responses following AAV-lacZ gene transfer.

**DISCUSSION**

Reports on immune responses to AAV vector-mediated transgene products have been conflicting. Many laboratories have demonstrated that the AAV vector can evade cellular immunity and induce a durable expression of the AAV-mediated transgene in vivo (7, 12, 13, 19, 20, 37). On the other hand, accumulating evidence indicates that in some circumstances, the AAV vector may initiate a detectable cellular and humoral immune response to the AAV vector-transduced neoantigen in vivo (8, 21, 22). Our findings demonstrate, for the first time, that immature DCs can take up AAV-lacZ and that the maturation of these cells may markedly reduce their susceptibility to the infection of AAV-lacZ.

Adoptive transfer of AAV-lacZ-infected immature DCs can significantly diminish AAV-lacZ-transduced muscle fibers in C57BL/6 mice. Adoptive transfer of various DCs infected with AAV and Ad vectors was performed to investigate the role of immature DCs in modulating the immune response in vivo (Table 1). In the first set of experiments, immature DCs were infected with either AAV-lacZ or Ad-lacZ. After being cultured in vitro in the presence of GM-CSF plus TNF-α for 3 days, these cells were adoptively transferred into C57BL/6 mice that had been injected with AAV-lacZ vector 10 days before (Table 1 and Fig. 2A). The mice were sacrificed 28 days later, and their muscles were isolated for X-Gal histochemistry staining. Consistent with our previous observation (12, 17), intramuscular injection of AAV-lacZ vector resulted in long-term expression of βGal in muscle fibers (Fig. 2A), with no βGal expression (top left panel). Coinfection of immature DCs with AAV-lacZ and wild-type Ad substantially enhanced lacZ gene expression in the immature DCs (top left panel), and this was further confirmed by X-Gal histochemistry staining (Fig. 1E). However, infection of mature DCs with either AAV-lacZ or AAV-lacZ plus wild-type Ad failed to induce detectable βGal expression (Fig. 1D, bottom left panel). Interestingly, βGal expression was also significantly reduced in Ad-lacZ infected mature DCs (bottom right panel). These results suggest that immature DCs can take up AAV-lacZ and that the maturation of these cells may markedly reduce their susceptibility to the infection of AAV-lacZ.

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FIG. 2. Impact of adoptive transfer of various DCs infected with AAV-lacZ and Ad-lacZ on AAV-lacZ-transduced muscle fibers of wild-type C57BL/6 and CD40L−/− mice. All mice were injected with AAV-lacZ in the left tibialis anterior on day 1. Then 5 × 10^5 of various DCs, generated from C57BL/6 mice and infected with different vectors as described in Materials and Methods and Table 1, were subcutaneously injected in the right lower quadrant of the ventral abdominal wall on day 10. (A and C) Representative macrographs of X-Gal histochemical stains of the left tibialis anterior that were harvested on day 28 after adoptive transfer of various DCs are presented. (B) β-Gal-positive fibers were counted in cross-sections of muscle, and the number was calculated as a percentage. The data represent the mean and standard deviation of the percentage of β-Gal-positive fibers from at least four mice. *P < 0.05 compared with the mice without adoptive transfer of virus-infected DCs. The abbreviation of each group is indicated in Table 1 and shows the recipient animals adoptively transferred with various DCs infected with distinct vectors. Magnification, ×45 (A) or ×40 (C).
neotransgene products have also been found in mice given AAV vectors encoding ovalbumin and herpes simplex virus type 2 virus glycoproteins B and D (8, 22), suggesting the production of persistent transgenic antigen stimulation in vivo. This would predict that the decline of AAV-transduced gene expression in vivo, if any, would occur in a chronic way due to the limited activation of T-cell immunity.

However, it remains unknown whether the elimination of AAV-lacZ-transduced muscle might result from shutting down the transgenic expression rather than killing the AAV-lacZ-containing cells. The muscle morphology of the mice given AAV-lacZ-infected immature DCs by adoptive transfer appeared to be fairly intact (Fig. 2A), in spite of the significant reduction of βGal activity in muscle fibers. The transgene expression of AAV in permissive cells correlates with the promoter activity and the phosphorylation state of the single-stranded D-sequence-binding protein (25). The previous studies have demonstrated that the cytomegalovirus promoter, which drives AAV-lacZ in our system, can be silenced by several factors in vivo independent of vector systems (10, 15, 16, 18). Although a substantial CTL response that could specifically kill the lacZ transgenic target cells ex vivo was observed from C57BL/6 mice given AAV-lacZ-infected immature DCs by adoptive transfer, we cannot rule out the possibility that other factors, such as cytokines, elicited by adoptive transfer of AAV-lacZ-infected immature DCs might interfere with the transgenic expression of AAV-lacZ in muscle.

Why did ex vivo AAV-lacZ-transduced immature DC, but not direct injection of AAV-lacZ into muscle, induce a cellular immune response? It has been shown that direct intramuscular administration of AAV-lacZ fails to induce significant infiltration of inflammatory cells (7, 12, 19, 20, 37) and to mobilize enough immature DCs into the injected sites to take up AAV-lacZ (17). Moreover, AAV-lacZ could induce a lower level of transduction of immature DCs. The experiments involving adoptive transfer of AAV-lacZ-infected DCs may artificially provide sufficient numbers of DCs bearing AAV-lacZ to initiate βGal-specific CTL response, which may not be achieved in mice given intramuscular injections of AAV-lacZ alone. However, this does not exclude the possibility that immature DCs might be recruited to the site to interact in situ with AAV vectors under some particular condition(s) such as delivery pathways and the presence of other inflammatory stimuli mobilizing the immature DCs. The evidence that intravenous injection of AAV-lacZ-infected immature DCs into C57BL/6 mice increased the percentage of IFN-γ-positive CD4+ T cells in spleen and lymph nodes (Supplementary Fig. 2) supports this hypothesis. Thus, the adoptive transfer of AAV-lacZ-transduced immature DCs may serve as a valuable tool to study the immune response against transgenic antigens in vivo.

FIG. 3. Induction of the CTL response to βGal in C57BL/6 mice after adoptive transfer of AAV-lacZ-infected DCs. C57BL/6 (A and B) or CD40L−/− (C) mice were first intramuscularly injected with AAV-lacZ and then given various DCs, infected with AAV-lacZ or Ad-lacZ, by adoptive transfer (Table 1). The lymphocytes isolated from the spleen were restimulated with Ad-lacZ in vitro for 5 days and analyzed for specific lysis using either Ad-lacZ-infected MC 57 (A and C) or pljβGal (B) transgenic cells as syngeneic target cells. All recipient mice were injected with AAV-lacZ. Results of one representative experiment of at least two are shown.

FIG. 4. Adoptive transfer of AAV-lacZ-infected DCs enhances the secretion of IFN-γ by cultured lymphocytes. The splenic mononuclear cells were isolated from the animals on day 28 after adoptive transfer of various DCs infected or not infected with AAV-lacZ (Table 1). A total of 2 × 10^6 cells were cultured in medium containing IL-2 (50 μg/ml) and βGal (10 μg/ml). The supernatants were collected 3 days later and assessed for IL-10 (A) and IFN-γ (B) by ELISA. Results are shown as the cytokine levels in pooled splenic mononuclear cells of four mice per group. The abbreviation for each group is described in Table 1 and shows the recipient animals given various DCs, infected with distinct vectors, by adoptive transfer. Results of representative experiment of two are shown.

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jection of mice with AAV-ova can elicit a more potent ovalbumin-specific CTL response than intramuscular injection does (8) implies that immature DCs may be recruited to interact in situ with AAV vectors in the intravenous pathway. These observations suggest that a threshold of AAV vector-transduced immature DCs may control the induction of the T-cell-mediated immune response to the transgene product. It is noted that DCs have recently been shown to take up antigens, not only through direct transduction by virus but also through cross-priming presentation by virus-infected targets (3, 6, 29). This study and other previous reports (8, 17, 21, 22) do not rule out the possibility that DCs might elicit a CTL response to AAV vector-transduced gene products through the cross-priming pathway. Further experiments are under way to address these mechanisms.

A sharp distinction was demonstrated between immature and mature DCs infected with AAV-lacZ in initiating βGal-specific CTL response to diminish AAV-lacZ-transduced fibers. Mice given AAV-lacZ-infected mature DCs by adoptive transfer failed to elicit a βGal-specific CTL response, whereas adoptive transfer of AAV-lacZ-infected immature DCs induced the generation of CTLs that markedly diminished the AAV-lacZ-transduced muscle fibers. Immature DCs are characterized by high endocytic and phagocytic activity and low expression of accessory signals for T-cell activation (2, 4, 11, 23). Maturation of DCs is always associated with the down-regulation of antigen uptake and the alternative expression of many functional surface molecules (2, 4, 11, 23, 26, 28, 31, 34, 41, 42). In fact, AAV-lacZ-infected immature DCs, but not mature ones, could express a minimal level of βGal, which was substantially enhanced by addition of wild-type Ad, suggesting that immature DCs are more susceptible to infection by AAV-lacZ than are mature DCs. The susceptibility of cells to infection by AAV-2 depends on its coreceptors αβ5 integrin and/or fibroblast growth factor receptor 1 and its primary receptor, membrane-associated heparan sulfate proteoglycan (24, 32, 33). In the absence of the coreceptor, for example αβ5 integrin, infection of cells with the AAV-2 vector fails to efficiently induce the transgenic expression (32). Immature DCs express high levels of αβ5 integrin, which mediates the phagocytosis of cells (2), although heparan sulfate proteoglycan and fibroblast growth factor receptor 1 remain to be examined. Interestingly, the expression of αβ5 integrin is significantly decreased during maturation (2); this may account for the reduced susceptibility of mature DCs to AAV-lacZ infection. Further investigation will be focused on elucidating the molecular mechanism of immature DCs taking up AAV-lacZ and processing the vector-transduced gene products.

Does AAV-mediated activation of CTL response require CD4+ T cells? Intramuscular injection of AAV-lacZ induces the production of IL-10 by lymphocytes but not that of IFN-γ (12, 17). It is reminiscent of Th2-dominated immunity to AAV-lacZ-transduced gene products (1). However, adoptive transfer of AAV-lacZ-infected immature DCs induced the production of IFN-γ, indicating that DCs preferentially polarized the development of Th1-mediated immunity, which may be responsible for the diminished βGal expression in AAV-lacZ-transduced muscle fibers. Activated Th cells express CD40L, and many studies show that its signaling through CD40 plays a critical role in enhancing the function of DCs to elicit cellular immunity (5, 27, 39). CD40L−/− mice given C57BL/6 mouse-derived immature DCs, infected with either AAV-lacZ or Ad-lacZ, by adoptive transfer failed to develop a βGal-specific CTL response to diminish AAV-lacZ-transduced muscle fibers. This indicates that vector-transduced DCs may in fact require additional signals in vivo via CD40-CD40L, despite the presence of high levels of surface B7 molecules prior to adoptive transfer (27). Alternatively, activation of third-party antigen-presenting cells via CD40-CD40L would be required to elicit a T-cell response. It is anticipated that the administration of anti-CD40L and/or anti-CD4 antibodies may effectively block a possible vector-mediated cellular immunity and lead to full recovery of gene expression transduced by AAV vectors.

In summary, our findings demonstrate that the AAV vector might be able to initiate a cellular response to its transduced gene products if sufficient immature DCs capturing the AAV vector and its transduced neoantigens are recruited. We propose that the AAV-carried transgene and the route of administration of the AAV vectors should be taken into the account when AAV vector-based gene therapy is designed for chronic genetic diseases. Moreover, one should consider that the treatment of patients with Ad-based vectors may prevent them from being treated with AAV-2 vectors carrying the same transgene as a result of the memory immune response, when a combination of different vectors carrying the same gene is used in clinical trials of gene therapy.

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REFERENCES

13. Flotte, T. R., S. A. Afione, C. Conrad, S. A. McGrath, R. Solow, H. Oka, P. L. Silverstein, and N. Bhardwaj. 1998. Immature dendritic cells phagocytose apoptotic cells via αβ5 integrin and/or fibroblast growth factor receptor 1 re-


