Adenoviral Vectors Targeted to CD40 Enhance the Efficacy of Dendritic Cell-Based Vaccination against Human Papillomavirus 16-induced Tumor Cells in a Murine Model

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ABSTRACT

Dendritic cells (DCs) represent a unique junction from which to initiate antigen-specific immunity. One of the most challenging obstacles for DC-based immunotherapy has been the means by which to convey tumor antigen-encoding genes to DCs. In this study, we show that adenoviral (or adenovirus, Ad) vectors targeted to CD40 by means of bispecific antibodies can enhance gene transfer to murine DCs. Moreover, we illustrate that this vector initiates phenotypic changes characteristic of DC maturation. To explore the in vivo potential of this strategy, we coupled this targeting approach with an Ad vector carrying the gene for a tumor antigen. In particular, the human papillomavirus (HPV) E7 antigen represents an attractive target for antigen-specific immunity of cervical cancer. Relative to DCs infected by untargeted Ad, DCs infected by AdE7 targeted to the receptor CD40 enhanced protection against HPV-16-induced tumor cells in a murine model. We have further established that this protection was both antigen specific and CD8+ T-cell dependent. Illustrating that Ad-modified DCs may be used in repeated vaccination, we report that preimmunization of animals with Ad infected DCs prior to E7 vaccination only moderately reduced vaccine efficacy. Finally, we have observed that CD40-targeted AdE7 can initiate partial therapeutic immunity in mice bearing established tumors. These findings suggest that gene-based vaccination of DCs with tumor antigens can elicit productive antitumoral immunity and that enhancements in gene transfer efficacy and/or DC maturation may facilitate this process.

INTRODUCTION

As a result of advances in the identification of tumor-specific and tumor-associated antigens, antigen-directed immunotherapy is emerging as a rational approach for the treatment of cancer. To this end, DCs are regarded as the predominant antigen-presenting cell of the immune system; the role of “mature” DCs in the activation of T cells is particularly relevant to immune responses against tumors (1, 2). In many instances, antigen presentation by DCs is regarded as a rate-limiting step in the generation of antitumoral immunity (2, 3). For these reasons, DCs represent a unique junction for intervention by antigen-specific vaccination strategies.

In this regard, strategies that use antigen-pulsed DCs have proven remarkably effective at protecting animal models from tumor challenge (2, 4–9). Among the methods described to pulse DCs with tumor-associated antigens, antigen-directed immunotherapy is emerging as a rational approach for the treatment of cancer. To this end, DCs are regarded as the predominant antigen-presenting cell of the immune system; the role of “mature” DCs in the activation of T cells is particularly relevant to immune responses against tumors (1, 2). In many instances, antigen presentation by DCs is regarded as a rate-limiting step in the generation of antitumoral immunity (2, 3). For these reasons, DCs represent a unique junction for intervention by antigen-specific vaccination strategies.

Received 1/21/00; accepted 8/1/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by an internal grant from the Division of Gynecologic-Oncology, University of Alabama at Birmingham and also by NIH Grants R01 CA74242, R01 CA68245, R01 CA68681-01, and U19 DK57858.

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3 The abbreviations used are: DC, dendritic cell; Ad, adenovirus; CAR, Coxsackie adenovirus receptor; Luc, luciferase; GFP, green fluorescent protein; HPV, human papillomavirus; MOI, multiplicity of infection; ATCC, American Type Culture Collection; Ab, antibody; mAb, monoclonal antibody.
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Viruses and Cell Lines. Ad carrying the gene for HPV E7 mutant in the retinoblastoma product (pRb) binding domain, indicated in the text as AdE7, was generously provided by Dr. Pradip Raychaudhuri (University of Illinois at Chicago, Chicago, IL; Ref. 28). Preparation of plaque titered AdLuc and AdGFP, carrying the gene for Luc and GFP protein, respectively, has been described previously (25). The C3 tumor cell line (a kind gift of Dr. Jan Ter Schegget, University of Amsterdam, Amsterdam, the Netherlands) was generated by transfecting C57BL/6 mouse embryonic fibroblasts with plasmids containing the entire genome of the HPV type 16 as well as the ras gene (29). B16 melanoma cells were obtained from the ATCC (Manassas, VA). Both C3 and B16 cells were cultured in DMEM supplemented with 4.5 g/l glucose.

CD40-targeting Conjugate. The anti-murine CD40 antibody FGK45 (30) was generously provided by Dr. Antonius Rolink (The Basel Institute for Immunology, Basel, Switzerland). The neutralizing murine hybridoma CD6.14 specific for the COOH-terminal, receptor binding knob domain of Ad serotype 5 fiber has been described previously (21). These hybridomas were used to generate hybridoma supernatants using Nutridoma (Boehringer Mannheim, Indianapolis, IN). Purification of antibodies and Fab fragments has been described (25). Bispecific antibodies consisting of the 1D6.14 neutralizing anti-Ad knob Fab fragment and the anti-CD40 Ab were prepared by chemical cross-linking with N-succinimidyl 3-(2-pyridyldithio) propionate as described previously (31). The conjugate of FGK45 mAB and 1D6.14 Fab is henceforth designated as Fab-anti-murine CD40.

Assessment of Phenotypic DC Maturation. For matutorial analyses, Abs were used directly to conjugate with FITC (PharMingen, San Diego, CA). These included: 3E2 (anti-CD54), 16-10A1 (anti-CD80), GL1 (anti-CD86), AF6-88.5 (anti-H-2Kc), AF6-120.1 (I-Ak), G155-178.95 (mouse IgG isotype control), R35-95 (rat IgG isotype control), and G235-2356 (hamster IgG isotype control). Anti-murine CD40, FGK45, was detected by the FITC-labeled goat antirat mAb (Jackson Immunoresearch Laboratories, West Grove, PA).

Bone Marrow-derived DCs. Bone marrow DCs were prepared as described previously in Inaba (32). Briefly, bone marrow was collected from femurs and tibias of C57BL/6 mice 4–8 weeks of age. Bone marrow cells were incubated with a mixture of antibodies directed against B220 (clone RA3-3A1/6.1), CD4 (clone GK1.5), CD8 (clone 53-6.72), and Ia (B21-2) using exhausted supernatants from hybridomas (ATCC). Subsequently, cells were incubated with rabbit complement (Cedarlane, Ontario, Canada) to deplete contaminating lymphocyte populations. Remaining cells were cultured in RPMI 1640 containing 10% FCS and 100 units/ml recombinant murine granulocyte/macrophage-colony stimulating factor (Peptotech, Rocky Hill, NJ). After 6 days of culture, loosely adherent DC clusters were collected and replated in 100-mm dishes for 3 h prior to infection. The purity of these DCs was established by the absence of lineage markers in flow cytometry analysis.

Preparation of Targeted Ad. To generate conjugate complexed virus, Ad was incubated with an optimal ratio of targeting conjugate as described previously (25). Briefly, Ad was incubated for 30 min at room temperature with Fab-anti-murine CD40 at a ratio of 30 ng:2.4×10^6 plaque-forming units in complete RPMI 1640 containing 2.5% FCS. Ad conjugated with Fab-anti-murine CD40 will be referred to henceforth as CD40-targeted Ad. For instances designated as untargeted Ad, virus was mock incubated with media containing no conjugate.

Infection of DCs for Assessment of GFP Gene Transfer. To assess the percentage of DCs transduced, cells plated in six-well plates were infected with untargeted or CD40-targeted AdGFP at an MOI of 10, 100, or 1000 in the presence or absence of conjugate for exactly 1 h at 37°C before unbound virus was washed away with PBS. Cells were subsequently incubated in RPMI 1640 containing 10% FCS (RPMI 10%). Alternately, cells were incubated with a constant MOI of 100 for a duration of 1, 6, or 24 h as indicated. After 24 h of incubation, cells were analyzed by flow cytometry for expression of GFP.

Infection of DCs for Maturation Analysis and Immunizations. Adherent DCs were incubated for exactly 1 h at 37°C under one of the following conditions: mock infection (DCs), CD40-targeted AdLuc (40AdLuc), untargeted AdE7 (AdE7), or CD40-targeted AdE7 (40AdE7). Subsequently, cells were washed with PBS to remove unbound virus, and RPMI 10% was added to each dish. After 24 h, pooled adherent and nonadherent cells were collected and used for either flow cytometry or vaccination experiments.

Prophylactic DC Immunization. Mice were administered a primary vaccination intradermally equal to the number of DCs indicated; 1 week later, a booster vaccination equal to half the dose of the primary vaccination was administered. Specifically, cell concentration was adjusted such that a 200-μl injection would constitute the indicated number of cells. This volume was distributed between 4 and 5 vaccination sites on the animal. One week after the booster vaccination, mice were challenged with tumor cells.

Tumor Challenge. Cells were released from culture vessels with trypsin and washed twice in PBS. Subsequently, mice were injected s.c. on the right flank with either 2 million C3 or 20,000 B16 cells as indicated.

T-Cell Depletion. To deplete CD8+ T cells in vivo, mice were i.p. administered 200 μg of purified mAb from the anti-CD8+ hybridoma 53–6.72 that had been purchased from the ATCC. Ab was administered relative to the primary vaccination on days −2, 1, 5, 10, 13, and 17. CD8+ depletion was validated by flow cytometry of splenic suspensions. On day 6, mice received a primary vaccination of 12,000 DCs infected as detailed in “Materials and Methods.” Subsequently, on day 7, a booster vaccination of 6,000 DCs was administered, and on day 14, a challenge with 2 million C3 was given.

Preimmunization of Mice with Ad-infected DCs. At 28 and 21 days after tumor challenge, mice were vaccinated with 25,000 and 12,500 DCs infected by AdLuc, respectively. At 14 and 7 days before challenge, mice received primary and booster vaccinations of 12,500 and 6,250 DCs, respectively, infected by either AdE7 or CD40-targeted AdE7, as indicated.

Vaccination against Established Tumors. Tumors were established by s.c. injection of C3 cells 3 weeks prior to the first vaccination. Only mice bearing tumors with a minimal volume of 100 mm3 at 3 weeks were advanced to therapeutic vaccination studies. Mice were size matched into four groups, with six mice per group corresponding to a group of unvaccinated animals or those vaccinated with DCs infected by CD40AdLuc (40AdLuc), AdE7, or CD40AdE7 (40AdE7). Mice were immunized with a dose of 200,000 DCs in a total volume of 200 μl on each of four weekly vaccinations. In particular, mice were vaccinated at sites distant from the tumor mass. Tumors were monitored for 15 weeks or until tumors had reached a volume of 1000 mm3, at which point mice were euthanized.

Statistical Analysis. The χ2 test was performed to analyze nominal data of tumor incidence from tumor protection experiments. The log-rank test was used to determine significance of therapeutic survival data in the Kaplan-Meier plot.

RESULTS

Retargeting of Ad to CD40 Increases Gene Transfer to Murine DCs. A limited availability of efficient strategies to deliver antigen-encoding genes to DCs has hindered gene-based DC vaccination strategies. We have illustrated previously high efficiency gene transfer to human DCs through targeting of Ad to CD40 by means of bispecific antibodies. Subsequently, we have transitioned this strategy to a murine context to allow evaluation of vaccine efficacy in an appropriate model system. Briefly, an activating anti-CD40 Ab, FGK45, was chemically conjugated to a Fab fragment of an anti-Ad Ab, 1D6.14, to generate a bispecific targeting conjugate. To illustrate that Ad complexed with this conjugate, henceforth designated as CD40-targeted Ad, could enhance gene transfer to murine DCs relative to untargeted Ad, delivery of the marker gene GFP by Ad was assessed by flow cytometry. As shown in Fig. 1A, CD40-targeted Ad demonstrated enhanced gene transfer relative to untargeted Ad at each MOI tested. At an MOI of 100, for instance, CD40-targeted Ad transduced 30% of cells, relative to 8% of cells by untargeted Ad. Importantly, these results reflect a strict 1-h incubation period of virus with cells before unbound virus was washed away. The enhancement in gene transfer by CD40-targeted Ad over untargeted Ad ranged from 3.6- to 9.9-fold. In contrast to our finding of low gene transfer with Ad in the absence of targeting, others have reported a high efficiency of gene transfer to DCs at similar dosage (17, 33). To reconcile our findings with these reports, we examined the possibility that more cells may be transduced after extended duration of viral incubation on transduction efficiency. As shown in Fig. 1B, extended exposure of cells to virus yielded a higher percentage of DCs transduced. In this regard, through extended incubation of cells with virus, untargeted Ad transduced...
upwards of 20% of cells by 24 h, yet CD40-targeted virus maintained a distinct and consistent advantage over untargeted Ad at all time points. These higher levels of gene expression after prolonged incubation with untargeted Ad may explain the findings reported by others. Collectively, these results illustrate that targeting Ad to CD40 increases the efficiency of gene transfer to murine DCs relative to untargeted vector.

**CD40-targeted Ad Phenotypically Matures Murine DCs.** We have described previously phenotypic maturation that accompanies infection of human DCs by CD40-targeted Ad (25). The essential role of maturity in the activation of T cells (1, 2) suggests that DCs modified by a CD40-targeted Ad vector might have enhanced potential in the context of immunizations. To evaluate whether a similar phenomenon accompanies targeting to murine CD40, DCs that had been infected with untargeted Ad or CD40-targeted Ad were compared with uninfected cells by flow cytometry (Fig. 2). Relative to uninfected cells, cells infected by CD40-targeted Ad enhanced expression of several markers associated with DC maturation, particularly CD40, CD86, and MHC II. Less substantial enhancements were observed for intercellular adhesion molecule 1, CD80, and MHC I. Minor changes were observed for cells infected with untargeted Ad, but these were less than that observed with CD40-targeted Ad. These findings indicate that targeting Ad to CD40 can mediate phenotypic changes that are associated with DC maturation.

**DCs Modified by CD40-targeted Ad Exhibit Enhanced Vaccination Potential.** To establish the efficacy of Ad-modified DCs for immunization, we have used the syngeneic C3 tumor model of HPV-induced neoplasms (34) and a functionally mutated gene for the E7 antigen of HPV within an adenoviral vector, AdE7 (28). To assess the potential advantage of CD40 targeting of Ad in a vaccination context, a dose-response curve was established to compare untargeted (AdE7) and CD40-targeted AdE7 (40AdE7) vectors. DCs infected ex vivo with an MOI of 100 were administered intradermally in primary and secondary vaccinations set 1 week apart. Specifically, DCs were

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**Figure 1.** Targeting of Ad to CD40 enhances the number of cells transduced relative to untargeted Ad. Murine bone marrow-derived DCs were infected with AdGFP either alone or complexed with Fab-anti-CD40 (A) for exactly 1 h at a MOI of 10, 100, or 1000 (B) for 1, 6, or 24 h at a constant MOI of 100 as shown. After 24 h of incubation, the samples were assessed for expression of GFP by flow cytometry. Results of representative experiments repeated in triplicate are depicted as a percentage of GFP-positive cells based on analysis of 10,000 cells.

**Figure 2.** CD40 targeting induces expression of DC maturation markers. DCs were infected by mock infection, untargeted AdLuc, or CD40-targeted AdLuc for 1 h at an MOI of 100 and subsequently incubated for 24 h prior to analysis. Samples shown indicate expression of CD54, CD80, CD86, CD40, MHC I, and MHC II as determined by flow cytometry. Isotype control antibodies are included for each receptor. A total of 10,000 cells were counted per condition.
tumors in unvaccinated mice is shown. Although unvaccinated mice developed C3 tumor masses, mice were challenged s.c. with 2 million C3 tumor cells. The percentage of mice bearing tumors at 6 weeks after tumor challenge is shown in this representative experiment. This experiment was repeated in duplicate. Analysis demonstrated significantly enhanced protection in mice vaccinated with 40AdE7 relative to mice vaccinated with AdE7 (P < 0.05).

Fig. 3. DCs infected by CD40-targeted Ad exhibit an advantage for in vivo vaccination over DCs infected with untargeted Ad. Mice were vaccinated by intradermal injection of graded doses of DCs infected by either untargeted (AdE7) or CD40-targeted AdE7 (40AdE7) as shown. On day −14, animals received a primary vaccination of 25,000, 12,500, or 6,250 DCs as shown. Subsequently, on day −7, mice were given a booster vaccination equal to half the dose of the primary vaccination. On day 0, animals were challenged s.c. with 2 million C3 tumor cells. The percentage of mice bearing tumors at 6 weeks after tumor challenge is shown in this representative experiment. This experiment was repeated in duplicate. Analysis demonstrated significantly enhanced protection in mice vaccinated with 40AdE7 relative to mice vaccinated with AdE7 (P < 0.05).

Fig. 4. DCs genetically modified by Ad vectors elicit antigen-specific immunity. Animals were left unvaccinated (Unvacc) or vaccinated by intradermal injection with DCs infected as follows: mock infected (DC), CD40-targeted AdLuciferase (40AdLuc), untargeted AdE7 (AdE7), or CD40-targeted AdE7 (40AdE7) as described in “Materials and Methods.” Except for unvaccinated animals, each mouse received a primary vaccination of 12,500 DCs and a booster vaccination of 6,250 DCs at 14 and 7 days prior to tumor challenge, respectively. One week after the booster vaccination, animals were challenged s.c. with 2 million E7-expressing C3 tumor cells or 20,000 B16 melanoma cells as shown. A representative experiment of two is shown, indicating the percentage of mice bearing tumors at 6 weeks.

**E7-based Vaccination Is Antigen Specific.** DCs impact the immune system through a number of antigen-nonspecific mechanisms (1). To establish that tumor protection was specific for E7 antigen, two avenues were investigated. First a control vector (AdLuc), carrying the gene for an irrelevant antigen, Luc, was used. Alternatively, a tumor line, B16 melanoma cells, negative for expression of the E7 antigen, was used in place of the C3 cell line for tumor challenge. As controls for nonspecific immune activation, DCs were left uninfected or infected with CD40-targeted irrelevant vector AdLuc. Mice were vaccinated with DCs infected with the indicated vector by a primary vaccination of 12,500 DCs, followed by a booster vaccination of 6,250 DCs 7 days later. A week after the booster vaccination, mice were challenged with 2 million C3 tumor cells or 20,000 B16 cells, as shown. Although unvaccinated mice developed C3 tumor masses, mice vaccinated with AdE7-transduced DCs did not develop tumors (Fig. 4). Importantly, the baseline percentage of mice developing C3 tumors in unvaccinated mice is <100%, as reported previously (5).

Notably, both unmodified DCs and AdLuc-transduced DCs imparted minor but not significant protection against tumor development. Alternatively, DCs transduced with AdE7, whether targeted or not, were unable to protect mice from challenge with antigen-disparate B16 melanoma. These findings illustrate that DCs genetically modified by targeted Ad generate immunity that is antigen-specific as defined by the transgene carried within the Ad vector.

**Depletion of CD8+ T Cells Abrogates DC-induced Immunity.** T cells play a prominent role in tumor rejection, and it is through T cells that DCs are believed to mediate their effects on antitumor immunity (35). To investigate the role of CD8+ T cells in the observed tumor protection, subsets of mice were depleted of CD8+ T cells during primary and booster vaccinations and subsequent tumor challenge with C3 tumor cells. Although both AdE7 and 40AdE7 conferred protection to challenge in undepleted mice, depletion of CD8+ cells entirely compromised the antitumoral effects of E7-based vaccination (Fig. 5). Thus, our findings confirm that the effector function of DCs infected either by untargeted or CD40-targeted Ad is mediated through CD8+ T cells.

**Preimmunization with Ad-infected DCs Does Not Prohibit DC-based Vaccination.** Immune-mediated clearance of Ad-transduced cells has prompted concern over the utility of Ad as a gene therapy vector, especially for repeated administration (36–38). To examine the potential that Ad-transduced DCs may compromise subsequent administrations, mice were preimmunized by primary and booster vaccinations of DCs infected by Ad carrying a gene for an irrelevant antigen, Luc. Subsequently, mice were administered primary and booster vaccinations of AdE7-transduced DCs at 1 and 2 weeks after preimmunization, respectively. To enhance the stringency of this experiment, the doses of DCs in primary and booster vaccinations for AdLuc-infected DCs were twice the doses of subsequent E7-modified DCs. One week after the final immunization, mice received a tumor challenge with C3 cells. In mice vaccinated with 40AdE7-infected DCs, mice that had been preimmunized with DCs infected by AdLuc exhibited tumor growth in 30% of animals, relative to complete protection in mice that had not been preimmunized (Fig. 6). These findings suggest that DCs may be administered on multiple occasions and yet still provide protection in a significant percentage of preimmunized animals.

**DCs Modified by Targeted Ad Extend Survival of Mice with Pre-established Tumors.** The initial goal of DC-based vaccinations in humans will likely be therapeutic, rather than prophylactic. We evaluated the capacity of Ad-modified DCs to mediate regression of sizeable established tumors in the murine model. Anticipating a more stringent challenge than prophylaxis, a larger vaccination dose was administered to elicit therapeutic immunity. Mice remained unvaccinated or were administered four equivalent doses of 200,000 Ad-modified DCs spaced at weekly intervals with DCs that had been infected by CD40-targeted AdLuc, untargeted AdE7, or CD40-
targeted AdE7, as indicated. As shown in Fig. 7, relative to unvaccinated animals, mice vaccinated with AdLuc-transduced DCs was not significantly distinct from unvaccinated animals. These findings confirm that genetically modified DCs can initiate an antigen-specific therapeutic immune response against E7.

DISCUSSION

The potential utility of genetically modified DCs is evidenced by their proposed applications in the treatment of infectious diseases, autoimmunity, allotransplantation, and cancer (1, 10). A significant hurdle to large-scale application of therapies using DCs will be a means by which to efficiently deliver antigen-encoding genes to these cells. In this regard, to explain poor infection of human DCs, we have reported previously a deficiency of the Ad binding receptor, CAR (25). Moreover, we demonstrated enhanced gene transfer to DCs by targeting Ad to an alternate receptor found on DCs, CD40. The receptor CD40 is expressed on DCs, macrophages, and B cells, as well as on endothelium and epithelial cells during inflammation (39). CD40 has numerous roles, including B-cell proliferation and isotype switching, as well as enhanced costimulation in macrophages. Foremost, CD40 plays a crucial role in the maturation of DCs. In particular, CD40 ligand, expressed on T cells, is believed to activate CD40 on DCs and promote their maturation to a more immunostimulatory phenotype, which in turn stimulates antigen-specific T cells (40–43). To exploit the expanding role of CD40 in DC function as an alternate Ad binding strategy, we have coupled a CD40-activating Ab with Ad vectors to achieve a high efficiency DC vector. Here, we describe the targeting of Ad vectors to CD40 on murine bone marrow-derived DCs and explore the utility of this approach in antigen-specific vaccination.

We have observed that Ad targeted to CD40 consistently demonstrated a greater magnitude of gene transfer relative to untargeted Ad. By comparison, our results reveal that untargeted Ad transduces a mere 8% of murine DCs at an MOI of 100; these findings are consistent with those of several reports (14, 44). In contrast, some investigators describe transduction efficiencies upward of 90% using targeted AdE7, as indicated. As shown in Fig. 7, relative to unvaccinated animals, mice vaccinated with DCs infected by CD40-targeted AdE7 were able to significantly delay continued growth and ultimately extended survival relative to either unvaccinated (P < 0.01) or AdE7 (P < 0.05) vaccinated animals. In contrast, tumor growth in mice vaccinated with AdLuc-transduced DCs was not significantly distinct from unvaccinated animals. These findings confirm that genetically modified DCs can initiate an antigen-specific therapeutic immune response against E7.
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A similar dose of untargeted virus (17, 33). To this end, it has been illustrated previously that upward of 80% of virions can localize to the nucleus of a cell within 60 min of infection (45); thus, it would seem that gene transfer that occurs on a longer time scale does so inefficiently. On these grounds, we have chosen a stringent 1-h infection period as a measure of rapid and efficient cell infection. To reconcile our findings with those of others, we reasoned that by extended exposure of DCs to Ad, higher levels of gene transfer might be achieved. In a comparison of different durations of Ad incubation, our finding of 20% of cells transduced by untargeted Ad at 24 h still falls short of the 90% reported by others. Nevertheless, our results do suggest that, much like the importance of the dose of virus used, the duration of incubation between virus and cells is an important, yet often unreported, parameter in the literature of Ad gene transfer to DCs. Importantly, the total percentage of cells transduced in the present murine system (30%) is strikingly less than that observed with human DCs (80%). In reconciliation of this fact, however, the overall enhancement in gene transfer by CD40-targeted Ad over untargeted Ad are 5-fold in human cells and 4-fold in murine cells. Thus, the fold enhancement in these two systems are, in fact, comparable, perhaps explained by the lower baseline gene transfer by untargeted Ad in murine cells, 8% in murine cells versus 14% in human cells (25).

The duration of incubation is perhaps inconsequential for ex vivo modification of DCs. Nevertheless, the practical advantages of ultimate in vivo DC transduction are fairly promising, especially in light of recent data suggesting that Ad targeted to CD40 can selectively transduce Langerhans cells of human skin. Accordingly, in vivo vaccination would eliminate the necessity for ex vivo manipulations to DCs, further increasing the ease and flexibility of this approach. High-efficiency vectors will become increasingly important because the duration of exposure of cells to injected virus may be limited under in vivo conditions. Perhaps most importantly, a high efficiency targeted Ad vector might have a distinct advantage in reducing the viral dose used in DC infection. Therein, the reduction of input viral dose may serve to minimize dose-related toxicity associated with Ad vectors (46–50).

We also provide evidence of phenotypic maturation in murine DCs infected by CD40-targeted Ad relative to untargeted Ad, a finding not unexpected given the CD40-activating capacity of the anti-CD40 mAb that was used in the targeting conjugate, FGK45 (41, 43, 51). Clearly, CD40 activation need not necessarily occur in the context of an Ad vector to mediate significant changes in DC phenotype and function. In fact, CD40 activation has been shown to potentiate any number of vaccination modalities (51, 52). For gene-based immunotherapy approaches, however, targeting Ad to CD40 can simultaneously increase much needed gene transfer efficiency of Ad vectors, with the prospective upshot of enhancing antigen presentation through DC maturation.

To establish whether DCs modified ex vivo imparted an advantage in vivo, we have compared the vaccination potential of DCs infected by untargeted and CD40-targeted Ad vectors using a murine model of cancers transformed by the human papillomavirus. Specifically, we have shown that Ad targeted to CD40 performed with greater prophylactic vaccination efficacy relative to untargeted Ad and in an antigen-specific manner. Our findings, however, do not indicate whether enhanced gene transfer or CD40-induced maturation is predominately responsible for the observed enhancements in vaccination performance.

Apprehension over the delivery of entire coding regions for oncogenes have prompted the use of peptide loading approaches for DC-based vaccinations (2, 10), and among these have included approaches directed toward HPV-E7 (51, 53–55). Nevertheless, the clinical application of peptide loading is likely to be encumbered by issues of practicality. Widespread application of allele-restricted peptides is limited in a human population with heterogeneous MHC alleles and further by the narrow range of epitopes provided by individual peptides (56). Such limitations are likely to be obviated through the use of gene-based modifications of epitopes. By delivery of the E7 gene in its entirety, DCs can present from among a vast array of potential epitopes that are appropriate for the MHC alleles of the recipient. The basis of E7 oncofugency has been defined (57, 58), and thus we have used a mutant rendered functionally inoperative in its oncogenic retinoblastoma gene product (pRb) binding domain (28). It is also important to recognize that E7 expression alone is not sufficient for malignancy (59), and further, that transformation is dependent upon continuous expression of E7 (60). The latter, in particular, is unlikely, given the short-lived expression by Ad vectors. Thus, in the context of the proper vector and with proper functional deletions to the gene of interest, the use of vector-delivered oncoproteins need not necessarily be viewed with skepticism. Because of safety concerns surrounding an Ad carrying the gene for wild-type E7 oncogene, we have not established the vaccination capacity of wild-type E7 relative to mutant E7. Conceivably, however, the small four amino acid deletion should be of little consequence for the vaccination potential.

Legitimate concerns have been raised about the utility of Ad vectors in a population that has been exposed previously to Ad (61, 62). Indeed, anti-Ad cellular immune responses have been recognized to severely compromise the duration of gene expression (36–38, 63–65). In particular, Jooss et al. (66) have shown that anti-Ad immune responses are a consequence of Ad transduction of DCs. In this regard, it would seem that DCs intentionally modified by Ad vectors would paradoxically serve as the vehicle for their own destruction. In contradiction to this presumption, however, several studies have highlighted the utility of Ad-infected DCs for vaccination despite prior immunization with infectious Ad particles (14, 15). We reasoned that rather than isolated Ad particles, preimmunization with Ad-infected DCs would more rigorously test the capacity of Ad-infected DCs for repeated administration. Our findings reveal that preimmunization with Ad-infected DCs does indeed decrease the immunization potential of subsequent DC vaccinations, yet a majority of mice still exhibit protection to tumor challenge. Several features might explain this counterintuitive finding. Foremost, Ad transgene expression in immunocompetent animals has been reported for at least 7 days prior to immune clearance (37). By comparison, the timeframe for both migration of DCs to lymphoid organs and interaction of DCs with T cells occurs much more rapidly (67, 68). Further to this end, it has been established that DCs undergo apoptosis after interaction with T cells (69); thus, it would seem that long-term expression of antigens is not requisite for initiation of a productive immune response. We hypothesized that activation of T cells by Ad-infected DCs may fall within a window prior to immune clearance of infected cells. Although our studies cannot conclude that repeated administrations will remain efficacious indefinitely, they do suggest that DCs modified by Ad might be administered in a series of boosters without entirely compromising their effectiveness. In this regard, the high efficiency of CD40-targeted Ad may serve to reduce the magnitude and/or number of doses of DCs necessary to attain a desired protective immunity before anti-Ad immune responses become insurmountable. For most gene therapy strategies, where long-term expression is indispensable, the fleeting expression of a transgene by Ad vectors is a conspicuous disadvantage. For DC-based immunizations, however, it would seem that even transient antigen presentation can effectively generate im-

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4 T. de Grauji, manuscript in preparation.
mune responses that would then be rendered enduring, not by the DCs but presumably through memory T cells.

The earliest applications of DC-based therapy will likely be therapeutic in nature. Despite the importance of cancer vaccines in this role, the effectiveness of other E7-based approaches in sizeable established tumors has not been demonstrated rigorously. We have vaccinated mice bearing palpable pre-established tumors with DCs infected by Ad carrying the gene for E7 or an irrelevant antigen. Our findings indicate that despite a significant prolongation in survival in animals vaccinated with DCs modified by CD40-targeted AdE7, a vast majority eventually succumb to the tumor. Several possible mechanisms might explain the failure of E7-based vaccination to mediate complete tumor regression. Foremost, the extended survival duration might explain an immune response is initiated but subsequently compromised or otherwise rendered ineffective. In particular, the tumor cells used in these experiments were not maintained under a selective pressure. It is possible that subpopulations of these cells did not express the E7 tumor antigen; alternatively, these cells may have undergone an “immunological escape” in vivo, much as human tumor cells tend to do (35). These findings suggest that an optimal vaccine will potentially incorporate several antigen genes within a single vector, thus minimizing the potential for such escape.

Of note, the expression of CD40 is not restricted to DCs, and with in vivo gene transfer, the potential exists for gene delivery to non-DC cell types. Unlike the delivery of genes encoding cytotoxic proteins used in some gene therapy applications, however, the relatively innocuous gene products of gene-based vaccination should not prove deleterious if delivered ectopically. Nevertheless, we propose to combine this targeting approach with DC-restricted transcriptional regulation to minimize ectopic gene expression. In contrast to the ex vivo methods described here, stability of a gene vector will be paramount for in vivo applications. Of note, we have demonstrated previously that retargeting of Ad with bispecific antibodies is stable for targeting in vivo (70). Nevertheless, we recognize the potential limitations of this strategy in large-scale vaccinations. For this reason, we are developing a virion with CD40 incorporated as a fusion protein with the Ad capsid. We anticipate that such a virion would exhibit further increased stability in vivo as well as minimize the potential for neutralizing anti-idiotypic antibodies directed toward the current Ab-based targeting conjugate.

Clearly, Ad is not the only means by which to modify DCs, in particular strategies described previously for pulsing DCs with tumor antigens or peptides represent alternatives that warrant comparison with the strategy described here. Also, we have not investigated the potential of other DC-maturing cytokines, such as tumor necrosis factor-α or interleukin 4, which could conceivably further augment the vaccination potential of this strategy. In summary, our findings indicate that Ad targeted to CD40 represents a high-efficiency, DC-potentiating gene delivery strategy that enhances the efficacy of DC-based immunotherapy strategies in an antigen-specific manner.

In the Division of Gynecological Oncology for their support of this work. We are indebted to Ronald Alvarez, Terri Pustilnik, and Edward Partridge for helpful discussions related to this work.

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