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CD40 -targeted adenoviral vectors to potentiate dendritic cells for antigen-specific vaccination.

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CD40-TARGETED ADENOVIRAL VECTORS TO POTENTIATE DENDRITIC CELLS FOR ANTIGEN-SPECIFIC VACCINATION

by

BRYAN WALTER TILLMAN

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2000

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ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

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Title CD40-Targeted Adenoviral Vectors To Potentiate Dendritic Cells for Antigen-Specific Vaccination

The complexities of tumor immunology suggest that innovative measures will be necessary to develop a vaccine for cancer. Increasingly, dendritic cells (DC) are emerging as a "central switchboard" of the immune system and thus represent an ideal junction from which to initiate anti-tumoral immunity. The most challenging obstacle has been the means by which to convey tumor Ag encoding genes to DC. Low efficiency gene transfer has encumbered conventional gene delivery approaches, and adenoviral (Ad) vectors are no exception. Reasoning this poor gene transfer as a potential defect in Ad entry, we have established a deficiency of the primary Ad binding receptor, Coxsackie adenovirus receptor (CAR), on human monocyte-derived DC (MDDC).

By means of bispecific Abs, we have circumvented the deficiency of CAR by redirecting Ad binding to an alternate receptor expressed on DC. The results have shown that by targeting Ad vectors to the cellular receptor CD40, gene transfer to DC could be increased considerably. More importantly, DC infected by this targeted vector underwent a "maturation" that enhanced their capacity to present Ags to other immune cells. These findings are commensurate with the established role of CD40 activation in native activation of DC.

To evaluate the in vivo utility of these genetically modified DC, we have coupled this retargeting approach with Ad vectors carrying genes for a defined tumor Ag. Human papillomavirus (HPV) is the causative agent of human cervical cancer, and thus, because expression of the HPV E7 gene is restricted to tumor cells, E7 Ag represents an ideal vaccine candidate. In an animal model of tumors induced by HPV, we have established prophylactic immunity against tumor challenge. Further, this strategy can initiate therapeutic immunity against established tumors that extends survival of tumor bearing animals. Finally, we have established that CTLs are a major effector cell in this antitumoral immune response. These findings suggest that gene-based vaccination of DC with tumor Ags can instigate productive antitumoral immunity and may have implications for the treatment of human cancer.

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SUMMARY

LIST OF ABBREVIATIONS

Ad	adenovirus
APC	antigen-presenting cell
CAR	Coxsackie-adenovirus receptor
CMV	cytomegalovirus
CTL	cytotoxic T lymphocytes
DC	dendritic cell
EGFR	epidermal growth factor receptor
FasL	Fas ligand
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony stimulating factor
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HPV16	Human papillomavirus type 16
LPS	lipopolysaccharide
mAb	monoclonal antibody
MDDC	monocyte-derived dendritic cell
MLR	mixed leukocyte reaction
MOI	multiplicity of infection
pRb	retinoblastoma tumor suppressor

RGD	arginine-glycine-aspartic acid
RLU	relative light units
TCR	T cell receptor

INTRODUCTION

Reapproaching Cancer Therapy

Primum non nocere "first do no harm"
-Hippocrates, Greek physician, *ca.* 400 B.C.

The justification for development of novel clinical therapies is found when conventional therapies have failed to provide satisfactory control over a disease entity. Certainly, the variable success of most conventional chemotherapy and radiation treatments for cancer would fit this criterion Indeed, the concept that a so-called cure should be nearly as grueling, debilitating, or lethal as the cancer itself is conspicuously antagonistic to the Hippocratic Oath of medicine. Perhaps most compelling, the carcinogenic nature of these so-called therapeutic agents is almost adversarial to the very premise of cancer therapy. For some, this tumorigenic potential may not be realized in their lifetime, but for young patients, the threat is real that the present therapy may serve only as a stay of execution before a new treatment-induced cancer arises.

Leukemia, in particular, is an increasingly significant consequence of anti-neoplastic therapy. Treatment-induced cases of acute myeloid leukemia, for instance, account for 10-20% of all cases (1). Besides secondary malignancy, numerous other side effects threaten the long-term health of patients undergoing chemotherapy, including cardiomyopathy, gonadotoxicity, growth impairment, obesity, and renal tubular dysfunction, among others (2). For most chemotherapeutics, alkylating agents and topoisomerase inhibitors, for example, it is their genotoxic profile which constitutes their effectiveness

as a chemotherapeutic agent; thus, nonspecific carcinogenesis is almost inevitable (1). Together, these aspects create a compelling case to endeavor novel therapies for this class of ailments.

An improved understanding of the upstream events involved in antitumoral immunity has underscored the central importance of APC. Recent evidence has suggested that through genetic modification of these cells, it might be possible to harness the potential of these immune mediators to enable tumor specific vaccination. In this regard, the discipline of gene therapy has risen to the forefront of cutting-edge medical therapeutics. The potential to initiate immune repsonses against tumor-specific Ags through genetically modified DC may soon provide a strict level of specificity that is so lacking with conventional pharmacologic agents.

Gene Therapy for Cancer

Gene therapy approaches for cancer have generally been encompassed in one of three conceptual groups: oncogene modulation, molecular chemotherapy, and genetic immunopotentiation. The objective of oncogene modulation is to suppress a gene that either has become mutated or exhibits dysregulated expression. To this end, dominant oncogene expression has been counteracted intracellularly through the use of antisense (3), ribozymes, or intracellular single-chain antibodies (3-5). Regardless, the chain of events that lead a cell to become "cancerous" are so heterogenous and multigenic (6, 7) that it is unlikely most cancers would be susceptible to treatment targeting one or a few genes or gene products. The second approach, molecular chemotherapy, is similar to pharmacologic chemotherapy, yet is distinct in that it restricts toxicity to tumor cells (8,

9). This approach encompasses delivery of a suicide gene or a gene for a prodrug activating enzyme to destroy a cancer cell independent of the cellular defect. Nevertheless, this approach is highly reliant upon cell-specific gene transfer and/or gene expression to minimize toxicity to normal tissues. In the final category, immunopotentiation approaches instigate an otherwise functional immune system against malignant cells. In particular, this approach may employ delivery of genes for immunomodulatory cytokines, tumor Ags, or costimulatory molecules to foster immune recognition of altered cells (10).

The success of both oncogene modulation and molecular chemotherapy strategies rests heavily on high-efficiency gene delivery. A particularly onerous obstacle for the field of gene therapy has been the seemingly unrealistic goal of gene delivery at 100% efficiency; therapies that fall short of this goal risk relapse of the cancer. In contrast, genetic immunopotentiation requires genetic manipulation of a handful of cells to generate systemic and potentially enduring antitumoral immunity.

A Brief History of Vaccines

The best means by which to visualize the future of genetic immunopotentiation is to understand where its conventional counterpart left off, namely in the field of vaccinology. The immune system is indisputably a powerful and dynamic system for the control of both infectious and tumorigenic entities. In this regard, vaccinations have proven to be an effective means to preemptively instigate the immune system against a number of foreign agents. In fact, aside from sanitation campaigns, vaccinations have

represented one of the most successful and cost-effective means by which to dramatically improve public health (11-13).

Vaccinations can be divided into classes of passive and active immunizations. Passive immunizations are employed for acute emergencies when insufficient time is available to develop a host's own immune system, such as by transfer of Abs to a recipient. Examples of passive vaccinations include antisera against spider bites, botulism, rabies, and snake bites (14). Importantly, however, passive immunizations do not generate memory response and, thus, would not protect an individual from a subsequent challenge with the foreign agent.

An alternate approach is active immunization, wherein the host immune system is exposed to attenuated, killed, or fractionated pathogens to elicit a lasting immune response. In this way the vaccine elicits protective immunity but also sustains a memory response to provide long-term protection. Today, a typical active immunization regimen in the United States includes vaccinations to hepatitis B, diphtheria, pertussis, tetanus, poliovirus, measles, mumps, rubella, and haemophilus influenzae (11). The first active immunization was described by Edward Jenner in 1796 (15). Noting that milkmaids who contracted cowpox were immune to the more deadly human disease smallpox, Jenner reasoned that intentional administration of cowpox might protect individuals from smallpox. Nearly 200 years later, smallpox had been eradicated from the human population. A century after Jenner, Louis Pasteur would apply attenuated pathogens in similar strategy, which he called vaccination (from the Latin vacca, meaning "cow") in deference to Jenner's work with cowpox. Soon Pasteur had developed vaccines to cholera, anthrax, and rabies (15). In general, killed or inactivated vaccines are required

in multiple doses and often only elicit humoral, Ab-based, immunity. Attenuated vaccines, contrast, provide for extended exposure to the pathogen, promoting increased immunogenicity in a single dose as well as improved cellular immunity. Despite their often superior immunization profile, attenuated vaccines carry risks, including contaminant pathogens, moderate disease-like symptoms, and the potential for reversion to virulence (14). One of the more novel developments in immunizations has been in DNA vaccines, wherein plasmid DNA encoding specific pathogen Ags is inoculated into skin or muscle of a recipient (15). Because neither keratinocytes nor muscle cells efficiently present Ag, it is likely that professional APC are involved. One particular advantage of this approach is that no organism is present in the vaccine preparation, thus eliminating the potential for attenuated organisms to revert to a virulent state. Also, DNA vaccines may allow prolonged expression of the Ag, promoting improved memory response and enduring immunity.

Despite the considerable success of vaccines in prevention of infectious diseases, no effective vaccines yet exist for malaria, tuberculosis, and HIV (16, 17). Moreover, in the 200 years since Jenner described the first vaccine, the world still awaits an effective vaccine for cancer. We now understand that tumors, much like infectious agents, employ a formidable arsenal to escape surveillance by the immune system (18). Moreover, the highly mutable state of most tumors creates a heterogenous mass of cells which may differ both in its antigenic makeup as well as susceptibility to therapy. The complexities of tumor immunology suggest that innovative measures will be necessary to develop a vaccine for cancer. The complex interactions of a multitude of immune components would seem to dash any hopes of mobilizing this network to therapeutic

potential. In spite of this, recent advances have begun to provide insight that seems to unify these components. Increasingly, the field of immunology points to the influential role of T cells and APC in both tumor immunology and the development of a vaccine for cancer.

The Interactions of T cells and APC

T lymphocytes constitute an arm of the immune system known as cellular immunity. The crucial role of T cells in the arena of tumor immunology has been highlighted by their ability to mediate protection against tumor development (18). Specifically, the ability of the T cell to identify cancer cells rests in its capacity to respond to tumor Ags. Much like B cells, T cells express molecules on their surface, known as the TCR, which recognize specific antigenic determinants (14). In particular, the Ags recognized by the TCR are short arrays of amino acids in the form of peptides in the context of MHC. As with the B cell receptor expressed by B cells, any particular T cell expresses a TCR specific for only a single epitope. In this way, a T cell is dedicated to recognition of a particular target. All told, the complete repertoire of T cells can recognize an almost infinite array of peptide combinations. In the absence of challenge by an immunogen, many of these cells are found in the circulation as naïve or unstimulated cells (19). Once a foreign agent has been controlled, however, memory T cells persist as a long-term and more quickly mobilized resident of the immune system. With each subsequent exposure to the cognate Ag, the T cell response becomes progressively larger (14). Unlike some other immune cells that can be stimulated by soluble Ags, B cells or macrophages for instance, T cells must recognize antigenic epitopes in the context of an APC (19, 20).

Specifically, the T cell receptor must bind to an epitope that is held by an MHC molecule on the surface of an APC. Importantly, these cells must also receive a concomitant costimulatory signal from a receptor of the B7 family, B7.1 or B7.2, for proper activation. Thus, the role of the MHC is to present antigenic peptides to T cells bearing cognate TCRs (14).

The loading of MHC with antigenic domains defines not only which Ags are presented but also to which immune effector cells they are presented. Specifically, evidence suggests that epitopes displayed on MHC class I and II are derived from different sources; class I MHC epitopes are intracellular Ags, and class II epitopes are extracellular Ags. In their basic role, class I molecules justify a cell's legitimacy as a normal uninfected and untransformed cell. Class I MHC is expressed on most every nucleated cell, and thus it can identify altered cells anywhere in the body. More specifically, presentation of intracellular gene products by MHC I can alert the immune system to intracellular pathogens such as viruses or bacteria or to cells in the process of becoming neoplastic (21). Essentially, proteins within the cell are degraded to short peptides by a cytosolic proteolytic system. After association with the MHC molecule, the new complex is transported from the rough endoplasmic reticulum through the Golgi complex en route to the plasma membrane. In contrast, class II MHC is employed for the presentation of Ags scavenged by phagocytosis or endocytosis from the extracellular environment and is expressed primarily on APC. The internalized Ag is degraded into peptides as it migrates through a series of intracellular compartments before pairing up with nascent class II MHC (21). Eventually, the resulting MHC-peptide complexes transit to the plasma membrane for presentation to T cells. Presentation of Ags on class I vs. class II ulti-

mately determines the T cell population to which they are presented; class I presents to CD8+ T cells, and class II presents to CD4+ T cells. Besides their different MHC affiliation, CD4+ and CD8+ T cells also mediate distinct effector functions (14). Activated, CD4+ T cells contribute to the local cytokine microenvironment, influencing either the humoral or cell-mediated branches of immunity depending on the signals received during their differentiation. CD8+ T cells, also known as CTLs, meanwhile, are directly involved in target cell lysis (14). Once a CTL has recognized a target cell by means of binding to Ag-associated MHC, cytotoxic granules are released in the extracellular space between the CTL and target cells. Among the most important agents within the granules are perforin and granzymes, which cause lytic pore formation and apoptosis, respectively, in the target cell. Further, Fas-ligand expressed on the surface of the CTL induces apoptosis in Fas-bearing target cells through direct cell-to-cell contact.

Historically, the role of the APC has been occupied by B cells and macrophages. Subsequently, the realization that dendritic cells (DC) express a much higher density of both MHC and costimulatory molecules has highlighted DC as the primary APC (22). In contrast to most other cell types, which present only endogenous Ags on class I MHC, DC may present Ags derived from the extracellular environment, a process known as "cross-priming," albeit inefficiently (23).

Rather than acting in a mere donor recipient fashion, DC-T cell interaction is instead viewed as an exchange of signals. Engagement of CD40 on the surface of DC by CD40-ligand on CD4+ T cells initiates a cascade that culminates in maturation of the DC (Fig. 1). In fact, DC are believed to perform as an essential intermediary between CD4+ and CD8+ T cells (24-26). While bound to the DC, T cells receive a dual signal from

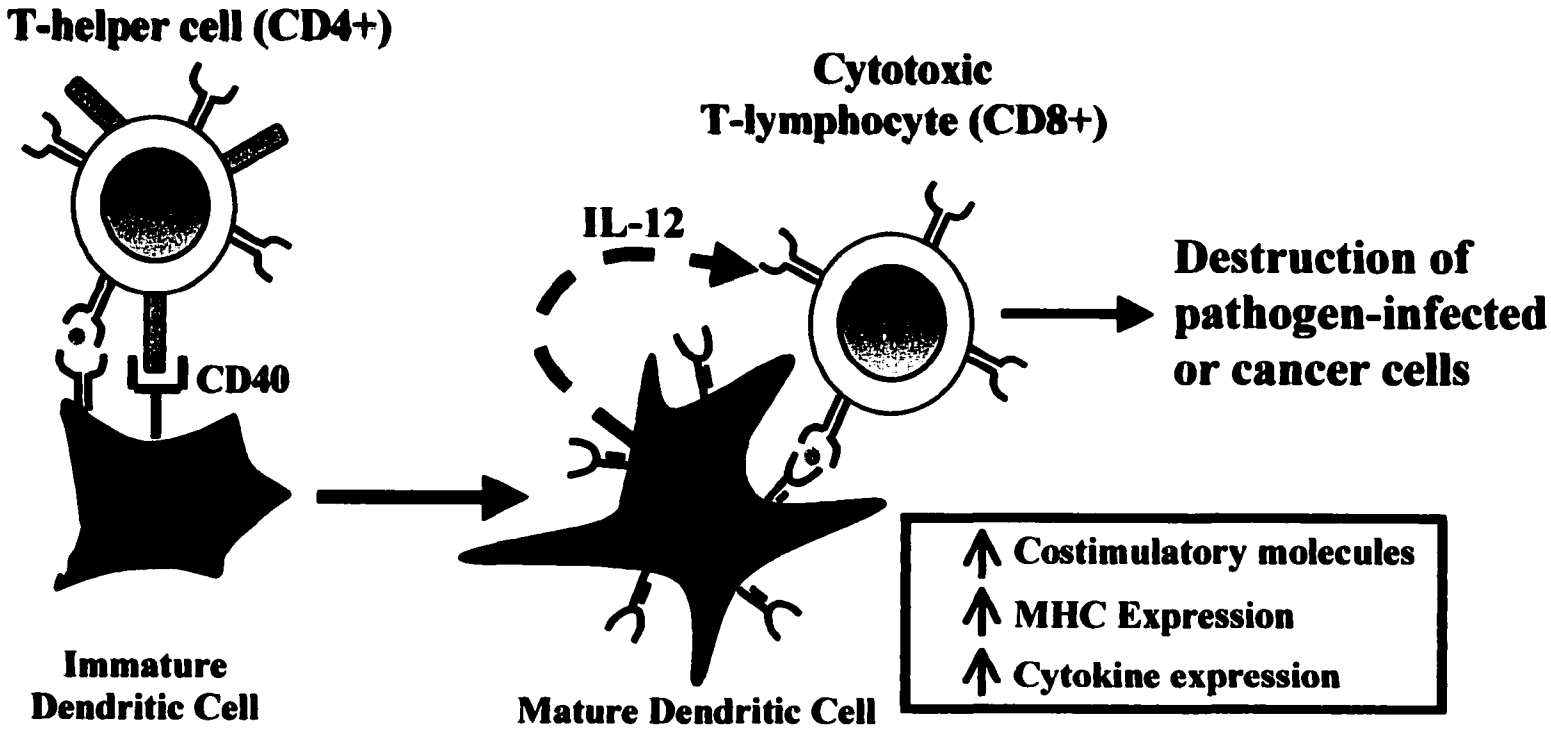


FIGURE 1. Stages in DC generation of T cell immunity. DC exist in one of two forms, each of which fulfills an important functional role. Immature DC are avid scavengers of extracellular Ags, while only mature DC can present these Ags effectively to CTL. CD4+ T cells induce maturation of DC through engagement of CD40 on the DC surface. Maturation increases expression of MHC, costimulatory molecules, and cytokines that are requisite for subsequent activation of CD8+ T cells.

molecules on the DC surface, namely MHC and costimulatory molecules. Importantly, failure of the T cell to receive a simultaneous signal from the costimulatory molecules B7.1 and B7.2 can arrest the cells in a state of anergy (14). From a functional standpoint, anergized T cells are paralyzed. Thus, concomitant expression of both MHC and costimulatory molecules is obligatory for proper activation of T cells. Besides direct cell-to-cell interactions, DC can significantly influence the cytokine milieu. Specifically, release of the cytokine IL-12 plays a crucial role in skewing the T cell component to a "Th1" response conducive toward cellular immunity (27).

The Hematopoetic Origin of DC

A number of key experiments have illustrated that DC are bone marrow derivative cells. In particular, the cytokine GM-CSF, traditionally associated with the development of monocytes and granulocytes from hematopoetic stem cells, has been linked to DC development (28). Even after cells have differentiated into one of these cell types, it appears that some lateral mobility still exists. Monocytes, for instance, can differentiate into immature DC, a process which can be observed *in vitro* following culture of monocytes with GM-CSF and IL-4 (29). To date, the distinction in function between lymphoid- and myeloid- derived DC has yet to be completely resolved. Nevertheless, lymphoid DC are believed to play a prominent role in deletion of autoreactive T cells (30). Myeloid DC, in contrast, are more commonly affiliated with an immunostimulatory capacity. Langerhans cells, the resident DC of the skin, are regarded as a classic myeloid DC (31). These cells are exquisitely apt at the uptake of Ags in transit across the epidermal barrier. These cells undergo a process of maturation following exposure to inflammatory mediators such as IL-1 (produced by other DC) or TNFα, bacterial cell

wall component LPS, or by cognate interaction with CD4+ T cells (32). During maturation, intracellular stores of MHC are translocated to the cell exterior, and surface expression of costimulatory molecules is increased. In accompaniment of maturation, phagocytic properties are dramatically diminished, effectively converting the cell from an "Ag scavenging" to an "Ag presenting" type. These cells migrate rapidly out of the epidermis and transit to secondary lymphoid organs, by means of the afferent lymph system (31). It is within the confines of the regional lymph node that DC activate Ag-specific T cells. Therein, evidence suggests that DC undergo apoptosis after interaction with T cells (33).

A growing body of evidence suggests that DC represent a central switchboard of the immune system, by interactions with T cells, B cells, and NK cells (22, 34-36). Collection of DC directly from tissues is time intensive, and even then the extent of cell recovery is too low to be useful in vaccination approaches. As an alternative, DC have been differentiated ex vivo from more readily accessible hematopoetic precursor cells. The heritage of DC believed most relevant to vaccination strategies is myeloid in origin; thus, precursors in this arena have been most intensely investigated. Generation of murine DC from blood-derived precursors was first described by Inaba (37) using the cytokine GM-CSF. Subsequently, it was noted that DC could be generated in larger quantities from lymphocyte-depleted bone marrow, again through the use of GM-CSF (28). Analogously, generation of human DC was described using CD14+ monocytes through exposure to GM-CSF and IL-4 (38, 39) Alternatively, through more cumbersome, rare human CD34+ precursors can be treated with GM-CSF and TNFα to generate DC (40).

Maturation of DC: An Essential Component of Immunostimulation

The maturation state of DC is known to play a particularly important role in their function as immune mediators (41). Immature DC are highly phagocytic, commensurate with their function as scavengers of foreign Ags. Nevertheless, these cells lack the panel of costimulatory molecules necessary to activate effector cells. The absence of these costimulatory molecules renders these cells tolerogenic rather than immunogenic. To this end, immature DC have been used to tolerize a host animal to autoimmune Ags or allogeneic Ags associated with allotransplantation. Perhaps most illustrative of the link between maturity and immune activation was the finding that administration of immature donor DC prior to allogeneic transplant can significantly prolong graft survival time; conversely, use of mature DC accelerated rejection (42). Maturation is accompanied by a dramatic upregulation of costimulatory molecules, including CD80 (B7.1) and CD86 (B7.2); during maturation, DC also lose their phagocytic activity (22). A number of factors are known to exhibit maturing potential toward DC, including the bacterial subunit LPS, the cytokine TNF-α, and the T cell surface marker CD40-ligand (22). Most of these stimuli have a physiologic significance to their maturational properties. LPS from gram-negative microbes, for instance, induces maturation so as to initiate a response to bacterial infection (22). It is clear that the maturation status of DC plays an important role in the ability of these cells to promote a productive immune response. As part of their counter-immunity arsenal, some tumors use IL-10 to suppress DC maturation or Fas ligand to induce DC apoptosis and thereby elude immune surveillance (22). Maturation of DC, most reliable when triggered by CD40-ligand, renders DC resilient to immunosuppressive cytokines such as IL-10 (43, 44) or TGF-β (45) and resistant to

apoptosis induced by Fas ligand (18, 22, 46, 47). Thus, maturation of DC has several advantages from a tumor vaccine standpoint.

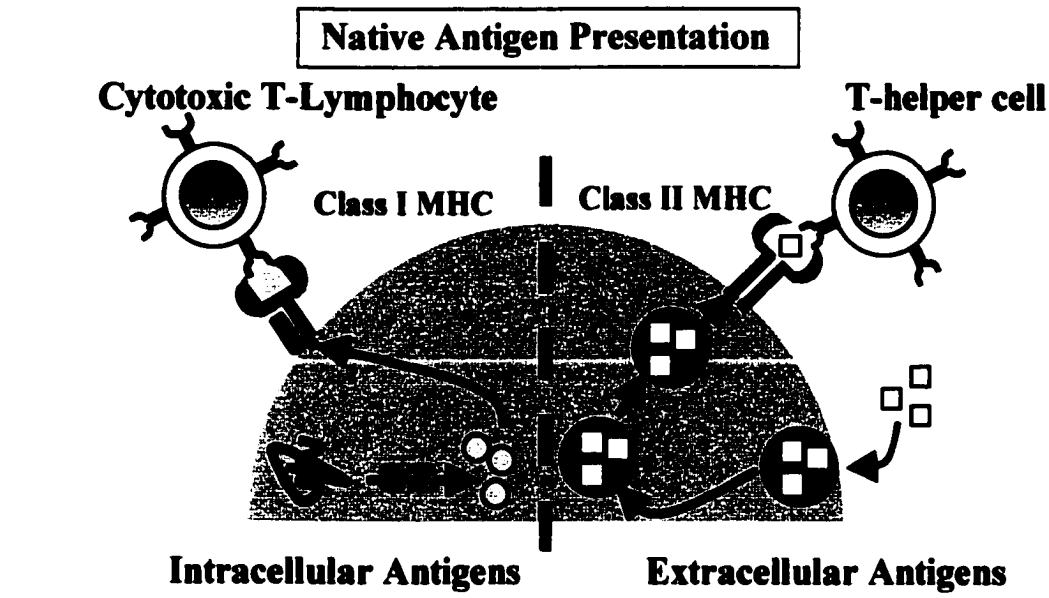
Immunology of Cancer: The Tumor Strikes Back

In many ways a cancer cell is similar to a cell infected by an intracellular pathogen, perhaps most analogous to a virally infected cell. Virally infected cells appear to be "self" for all practical purposes. Nevertheless, the foreign viral Ags presented in the context of host MHC class I reveal the presence of intracellular abnormalities. It is these viral Ags that are recognized as "nonself" and toward which a protective immune response is mounted. Likewise, in the case of cancer, these cells often express minor yet distinct antigenic profiles compared to normal cells (14). Some may express residual genes of an instigating tumorigenic pathogen, such as viral Ags. Others may reactivate genes that had been expressed early in development but which are no longer expressed on normal adult tissues. Alternately, the mutations that accompany transformation of a cell, including those in tumor suppressor genes, growth factor receptors, and cell cycle proteins, among others, can serve as early tell-tale signs of a cell gone awry (17). This may render the tumor "apparent" as nonself, either in part or in entirety. In fact, clearance of transformed cells by the immune system is not uncommon (14). This fact is highlighted by the increased incidence of some cancers among immunosuppressed individuals (48-50). Nevertheless, tumors with distinctive antigenic profiles may yet grow unchecked despite an otherwise competent immune system.

Recent evidence suggests that tumors actively escape surveillance or eradication by the immune system (17). Tumors may undergo antigenic modulation in which tumor

Ags undergo endocytosis or shedding to conceal their transformed phenotype. Alternately, by downregulation of MHC class I (17), tumors may disguise antigenic intracellular gene products. Unlike professional APC, tumor cells often do not express costimulatory molecules. For this reason, tumor cells cannot themselves activate naïve tumor reactive T cells; in the absence of costimulatory signal they may even tolerize T cells, further exacerbating the deficient immune response (17). The latter scenario was a compelling incentive to pursue APC as a therapeutic agent. In a more preemptive manner, tumor cells may induce apoptosis of reactive T-lymphocytes; a feature noted in examples of melanoma (51), colorectal cancer (52), hepatocellular carcinoma (53), breast cancer (54) , astrocytoma (55), and multiple myeloma (56), among others. Because, T cells are a major component of antitumoral immunity, their destruction not unexpectedly leads to tumor progression. Likewise, tumors may induce apoptosis in APC both by direct contact and by tumor-derived factors. A positive correlation between tumor-infiltrating DC with improved tumor regression has been illustrated for a number of tumors, including tumors of the lung, breast, stomach, and bladder (57). These findings are bolstered by evidence that increased numbers of DC were noted in benign tumors, yet decreased numbers of DC were found in many cases of malignant lesions (58). Finally, relative to the conspicuously foreign nature of most bacterial or viral pathogens, for instance, the "self" nature of cancer tends to preclude a vigorous inflammatory response. Inflammatory mediators are a crucial component T cell activation, particularly as relates to Ag presentation, and thus, the microenvironment surrounding the tumor may, counterproductively, promote tumor tolerance (17).

A primary reason that conventional vaccines have been ineffective against cancer may stem from their failure to activate the proper branch of the immune system. In this regard, the immune response has two arms: a humoral response producing antibodies and a cell-mediated response to destroy altered self cells. Therein, because the soluble Ags of conventional vaccines activate B cells, these preparations are ideal for generating a humoral immune response; the resultant Abs are quite effective at neutralizing and destroying extracellular pathogens (21). The role of Abs in the control of virally infected or cancerous cells, however, is greatly overshadowed by that of T cells (17). Thus, an optimal vaccine design for cancer or virally infected cells would instead enable T cell activation, particularly that of CTLs. Conventionally, soluble Ags are presented in the context of class II MHC, resulting in CD4+ T cell response (Fig. 2). Although dendritic cells are unique in their ability to cross-present extracellular soluble Ags in the context of class I MHC, this process is highly inefficient, and thus crucial antitumor CD8+ T cells are left without activation (21). The inability of DC to efficiently present conventional soluble vaccine Ags to CTLs explains why they have proven relatively ineffective to generate an antitumor CTL response (21). To instead elicit a class I-restricted immune response, several alternative immunization strategies have been suggested. Because particulate Ags can undergo cross-presentation to class I MHC with 1000 to 10,000 times the efficiency of soluble Ags (59, 60), new vaccines have been developed in a particulate format (21). Alternatively, vectors that invade DC intracellularly, both bacterial and viral, can deliver Ag genes that will be subsequently expressed and presented in the context of class I MHC (21). In fact, some vaccines prepared from attenuated pathogens capitalize upon the feature of intracellular gene expression as one of their key advantages



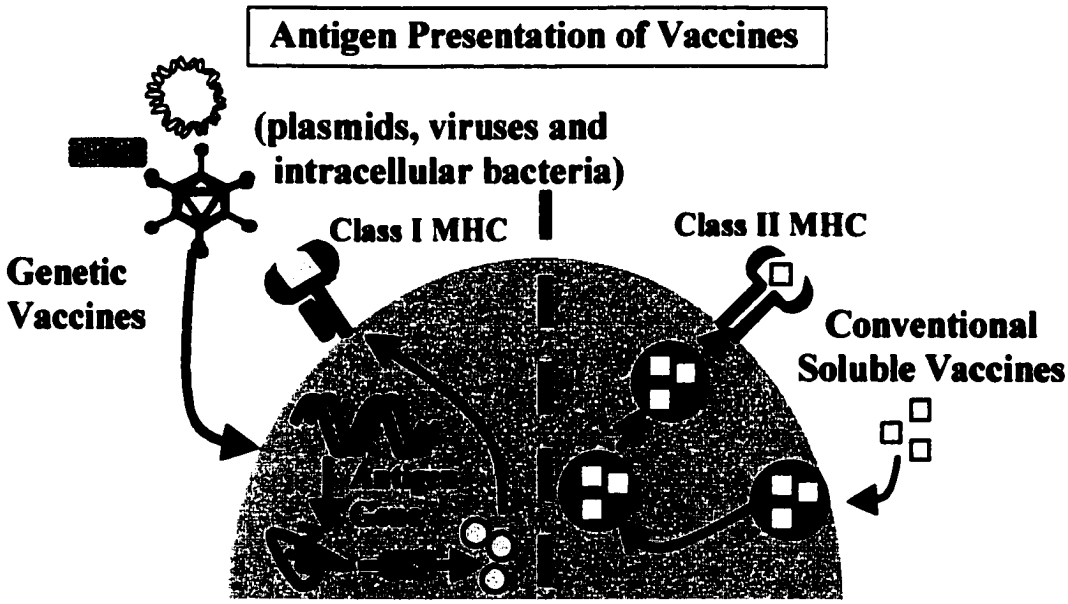


FIGURE 2. Gene-based vaccination enables activation of CD8+ T cells. CD8+ T-cells are an essential component of immunity against pathogen-infected or cancer cells. Their activation is dependent upon Ag presentation in the context of MHC class I. Class I Ags are derived from intracellular compartments, class II antigens are extracellular. Accordingly, conventional soluble (extracellular) vaccines load predominately class II MHC and do not adequately activate class I-dependent CD8+ cells. In contrast, genetic modification of DC creates an intracellular pool of Ag for presentation on class I MHC and acts as a more potent activator of CD8+ T cells.

over killed or fractionated pathogen vaccines. For many pathogens, however, the development of attenuated versions is impractical, or the risk of reversion to virulence is too dangerous (15). Increasingly, the potential of modified APC in CTL activation is becoming evident; therefore, through the use of DC, it should be possible to transcend the obstacles that encumber conventional vaccine strategies.

Immunotherapy

In recent years, the simultaneous advancement of several technologies has made the concept of gene-based immunotherapy a more realistic option in the treatment of numerous diseases. Foremost to the immunological aspect was the development of a unified scheme for the interaction of different classes of immune cells in a cohesive fashion. Central to this schema was the initial characterization of DC by Ralph Steinman in 1973 (61). In the 25 years since, it has become evident that dendritic cells act as a central switchboard of the immune system, governing the development of productive immune responses as well as tolerance. In this way, DC create a microenvironment conducive to interaction of T cells with their cognate epitopes as well as a milieu of cytokines that facilitate their effector functions (22). To date, such strategies have endeavored to either tilt the cytokine balance so as to create a generally antagonistic environment toward tumor cells or, alternatively, to trigger Ag-specific immunity against distinctive tumor markers. The advent of technologies to identify tumor-specific Ags (17, 21) has enabled the design of therapies with minimal toxicity to normal cells.

With the understanding that immunity played a major role in tumor recognition and destruction, efforts to skew the immune response toward a tumor-hostile environment

led to development of cytokine-based therapies. Specifically, a fraction of tumor cells, immune effectors, or third party cells (17) could be genetically modified to express limited quantities of immunomodulatory cytokines (62). By expression of a cytokine locally from a finite number of cells, toxicity could be minimized compared with conventional systemically administered cytokines (10, 63). Approaches using IL-2 (64), IL-12 (65, 66), and IL-18 (67), among others, have met with promising results in mediating tumor regression. IL-12 has been particularly successful, probably because of its ability to promote a CD4+ Th1 response, culminating in activation of APC and CTL. Another particularly effective cytokine therapy has been the use of GM-CSF. The role of GM-CSF in promoting DC differentiation (17) pointed once again toward APC as a promising tool for cancer immunotherapy. Nevertheless, the complex interactions between cytokine signals suggest that cytokine therapy might inadvertently antagonize the endogenous cytokine balance in favor of tumor growth. The nonspecific consequences of cytokines have prompted the development of more tumor-specific therapies.

Tumor Markers: Picking Cancer Out Of A Crowd

One Achilles heel of tumors is the expression or overexpression of gene products either not found or not found appreciably in normal cells. It is in this way that tumors might be distinguished from normal cells. Two classes of tumor markers are tumor-specific and tumor-associated Ags (14, 17). Tumor-specific Ags include those proteins wholly restricted to expression on tumor cells. Examples might include mutated self genes such as mutated ras or p53, or gene products of a viral instigator like human papillomavirus E6/E7 gene products. The tumor-associated class, in comparison,

includes Ags that are not unique to cancer cells but, rather, are over expressed on the malignant cells; examples include the melanoma-associated Ags Mart-1 and gp100 (17). Also included in tumor-associated Ags are gene products of reactivated embryonic genes, such as alphafetoprotein (AFP) and carcinoembryonic Ag (CEA).

DC in Therapy of Cancer: Genetic Engineering of DC

A greater understanding of DC basic biology and their interactions with other cells of the immune system has suggested DC as a rational conduit by which to instigate antitumoral immunity. In particular, it was believed these potent immunostimulators would augment the vaccination potential of an Ag. Indeed, adoptive transfer of genetically modified DC has shown to be significantly more effective than currently employed direct intramuscular injection of DNA for vaccinations (68). Even without modification, DC have been shown to confer a therapeutic effect, as evidenced by the moderate antitumoral effect of DC mobilizing cytokines such as FLT3-ligand (69, 70). Central to the use of ex vivo-modified DC was the identification of means to generate large numbers of DC ex vivo from blood and bone marrow (71).

Currently, among the most challenging obstacles has been the means by which to convey Ags to the surface of the DC in the proper context of MHC and costimulatory molecules. Early loading mechanisms have included pulsing with tumor-specific peptides (72) and gene transfer to DC (71). When tumor Ags are undefined for a tumor of interest, "feeding" DC apoptotic tumor cells (73, 74), and coincubating DC with tumor lysates, tumor-derived mRNA, or tumor-stripped peptides (75) have been employed. Alternately, DC and tumor cells may be physically fused to create a chimera that

presents Ags derived from the tumor portion on the Ag presentation machinery of the DC component.

Ultimately, the ideal immunotherapy will be Ag-specific because the above strategies risk the potential for autoimmunity by breaking tolerance to self Ags. Advances in the identification of tumor-specific and tumor-associated Ags have now engendered Ag specific immunotherapy as a rational treatment strategy. The primary means to load DC with defined Ags have included pulsing DC with defined antigenic peptides and gene-based Ag loading. The approach of loading of DC with individual tumor Ag peptides has met with a great deal of success in the setting of animal models of homogenous MHC background. In particular, adoptively transferred DC pulsed with Ag have shown utility in establishing peptide-specific prophylactic and therapeutic immunity (5, 18, 76, 77). Nevertheless, the effectiveness of this procedure is likely to encounter practical dilemmas when introduced into humans. Given the extensive heterogeneity of MHC alleles in the human population, the binding of these allele-dependent peptides would be extremely variable (21). Second, these peptides provide only a fraction of the potential epitope reservoir for presentation (15). Reports that tumors can change their antigenic profile (17, 78, 79) should be compelling grounds to target multiple epitopes (17, 78, 79). Moreover, the extra expenditure to derive peptides seems redundant because the DC itself is endowed with the capacity to derive its own array of epitopes from a given Ag. Finally, peptide approaches have shown their potential to counterproductively tolerize rather than activate immunity, through binding to non-APC cell types (80, 81).

A strength of gene-based strategies is that they do not require prior knowledge of HLA haplotype of patients or of T cell epitopes (71). Initially, delivery of open reading

frames for Ag-encoding genes has been viewed with disfavor, namely by citing that some of the derivative gene products are themselves oncogenic. While this is indeed the case, it also true that for many of these genes the basis of their oncogene activity has been defined, and thereby through targeted deletions in these genes, their oncogenic capacity can be rendered nonfunctional (82). Thus, the arena of gene-based Ag loading presents features that might prove advantageous in immunization strategies. Nonetheless, the current lack of vectors with efficient gene delivery to DC will ultimately come to bear upon the success of Ag specific immunotherapy strategies. To this end, the second major development for gene-based immunotherapy has been in the arena of gene transfer. A greater level of understanding of basic vector biology as well as the capacity to selectively target gene vectors should enable efficient gene delivery to and expression by APC.

Genes and Gene Transfer

It is increasingly evident that, provided the correct context, genetic elements can be subject to a great deal of regulation, imparting an advantage over conventional pharmacologic agents (83). Modern gene therapy has been the accumulation of numerous advances; nonetheless, several major developments have proven to be fundamental. The initial concept of discrete hereditary units was first advanced by Gregor Mendel in the 1860s (84). In a finding that would herald modern gene transfer applications, Oswald Avery demonstrated in 1944 the transfer of genes from one strain of bacteria to another, effectively imparting the recipient with new morphological properties (85). Nevertheless, it was not until the structure of DNA had been defined in a seminal article by

Watson and Crick in 1951 that even the faintest promise of true, controlled genetic manipulation could be envisioned (84). The development of calcium phosphate coprecipitation with DNA gave rise to marked enhancements in gene transfer compared with unmodified DNA or DNA-dextran complexes (86, 87). In perhaps the earliest use of viruses for gene transfer, bacteriophages were used to transfer DNA into bacteria (88), followed by viral gene transfer to animal cells (89, 90). In particular, viral vectors have exhibited vastly improved efficiency over nonviral methods.

Vector technology is one of the limiting agents in gene therapy, as highlighted in a report from the Orkin-Motulsky commision, initiated by the United States Congress (91). In particular, the report noted a deficiency in the arena of gene transfer vectors. Indeed, in potential applications ranging from cystic fibrosis to muscular dystrophy, the candidate genetic elements have been well defined, but the vector aspect remains the limiting agent for clinical gene therapy (92, 93). By far the biggest obstacle to gene therapy efforts is the necessity for high-efficiency and target cell-specific vectors for these payloads. Years before, Edward Tatum had articulated an early prediction about the use of viruses as gene vectors (94) :

> Finally, it can be anticipated that viruses will be effectively used for man's benefit, in theoretical studies in somatic-cell genetics and possibly in genetic therapy. . . . We can even be somewhat optimistic on the long-range possibility of therapy by the isolation of design synthesis, and introduction of new genes into defective cells of particular organs.

The early history of gene therapy introduced several physical methods to introduce DNA into cells. Nonetheless, attempts to employ such nonspecific and inefficient methods for use in human gene therapy were regarded as reckless (95). Viruses promised the potential for higher efficiency gene transfer along with a glimmer of hope that

they might one day be targeted to specific cell types for calculated genetic modification of tissues. With an ever-expanding understanding of viral biology, vector development continues to strive toward the ideal goal of a targetable, injectable gene therapy vector.

The advent of therapies that are designed to modify cell function through genetic modification has increased the demand for targeted gene delivery. Foremost, ectopic gene delivery increases the risk that expression of gene products might dysregulate normal cell function. When toxin- or prodrug-activating enzyme genes are employed, for instance, expression of genes in nontarget cells can threaten toxicity to normal tissues. For these reasons, restricted expression to a cell type of interest becomes paramount. Two means by which to achieve this goal are transcriptional and transductional targeting. Transcriptional targeting broadly encompasses strategies to restrict gene expression to the cells of interest. In the simplest incarnation of transcriptional targeting, the gene of interest is fused to a promoter for which the activity is restricted to certain tissue types (83, 96). Thus, despite widespread dissemination of a gene, expression would occur only in cells conducive to the tissue-specific promoter. Despite initial promise, this strategy has met with disappointing results, which include poor expression, leaky expression in nontarget tissues, or even loss of tissue specificity in the context of a viral genome (83, 97). Currently, resolving these issues has become a topic of much interest in the gene therapy arena, and a redesign of these promoter systems has begun to resolve these issues (98). As an alternative to transcriptional targeting, regulation might be positioned upstream of gene expression, namely over the cells to which the gene is delivered in the first place. To that end, transductional targeting is gene delivery that occurs only to the

cells of interest. The use of viruses for targeted gene therapy has awaited key developments in the understanding of viral biology.

Viruses, in their simplest embodiment, consist of genetic material in the form of RNA or DNA encapsulated within a protein coat. These capsids may further be characterized by the presence or absence of a lipid membrane around the capsid, referred to as enveloped or nonenveloped viruses, respectively. In any case, the virus is an obligate intracellular parasite and cannot complete its life cycle without having first infected a living cell.

Targeting viral vectors to promote specific gene transfer to a cell population of interest became an early goal in gene therapy. Retroviruses were the earliest vectors to be targeted by modifications to the viral surface (99). Nonetheless, it was soon realized that modifications to the envelope glycoproteins for targeting purposes had deleterious effects on viral entry (99, 100). As a consequence of the intimate association between the binding and cell entry domains and manipulation of the binding region can impair conformational changes necessary for activation of the fusion domain (101-103). Alternatively, the targeting potential of another viral vector, Ad, was investigated. In particular, Ad exhibits spatially distinct cell binding and internalization domains that have proven conducive to modification for targeting.

Development of Adenovirus as a Gene Therapy Vector

Ad is a double-stranded DNA virus, the genome encased within an icosahedral protein shell (104). Ad viruses cause a variety of mild and self-resolving pathological states including conjunctivitis, respiratory disease, and gastroenteritis (105, 106). First

identified in human adenoid tissue in the 1950s (107), several properties have prompted the popularity of the adenovirus as a gene therapy vector. Unlike retroviral vectors, the Ad virion is stable after systemic administration. Moreover, its high titer vector production facilitates its use in large-scale clinical applications. Finally, Ad vectors can infect a wide variety of cell types regardless of whether the cells are dividing or not, thus expanding their potential utility in clinical applications.

Foremost in the development of Ad as a gene therapy vector was the development of a replication-incompetent virus. Specifically, the virus was rendered incapable of initiating a replication cycle through deletion of a key viral gene. The E1 gene represented an ideal candidate for such deletion because not only are its gene products upstream of a cascade of genes involved in replication, but it is also recognized as a oncogene (108). For propagation of these vectors, packaging cell lines have been developed which express the missing gene products and facilitate restricted replication of the virus within the line (109, 110). To date, the E1-transformed embryonic kidney cell line 293 has been the classic packaging cell line for Ad vectors. Subsequently, it has been recognized that homologous recombination between the packaging line and the vector genome can generate replication competent Ad (RCA). New packaging lines carefully designed to minimize overlapping sequences between the packaging line and recombinant vectors have almost completely eliminated the potential for RCA (111). Various shuttle systems that rely on homologous recombination between a plasmid carrying the transgene of interest and an Ad genome rescue vector in mammalian cell culture have been developed to facilitate generation of recombinant Ad vectors (112). Moreover, newer systems incorporate a bacterial intermediate to simplify the entire process further (113).

While enabling its utility in a variety of scenarios, the broad tropism of the Ad severely compromises the specificity of this vector for a cell type of interest. In many instances, the potential for ectopic gene transfer can have enormous consequences, as illustrated by the hepatic toxicity observed in the HSV-tk enzyme prodrug trial for therapy of colon cancer by Van der Eb (114). Additionally, the liver has been reported to sequester upwards of 90% of the vector dose when administered intravenously (115, 116). Both sequestration of vector and infection of nontarget tissues necessitate the use of higher vector doses. These larger doses, in turn, increase the potential for toxic side effects. To compensate for the tropism issue, Ad vectors were administered to defined body compartments, with the reasoning that anatomical barriers would preclude gene transfer to tissues outside the compartment. Subsequently, it was realized that despite this approach, Ad could still be detected in distant tissues (114, 117). Fortunately, an understanding of the Ad entry pathway suggests means to overcome the promiscuous tropism of Ad vectors.

Redirecting Ad Tropism

A starting point for rational targeting of Ad vectors is the Ad entry pathway (Fig. 3). Ad adheres to target cells by means of the trimeric fiber protein which extends from each of the twelve vertices of the Ad capsid (104). Specifically, the carboxy-terminal region of the fiber, also known as the knob domain, has been shown to interact with a receptor known as the Coxsackie-Ad receptor (CAR) (104, 118). After-

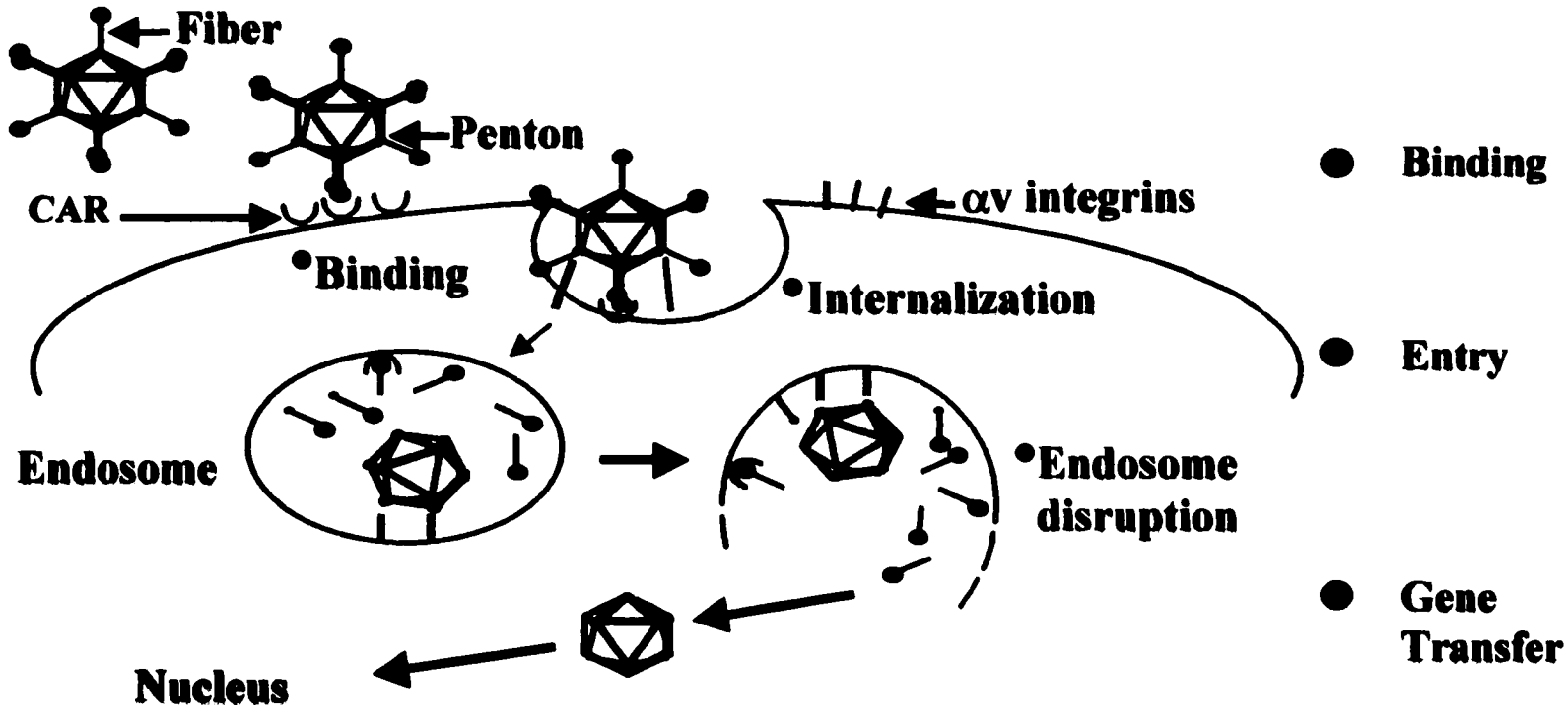


FIGURE 3. Ad entry pathway. During viral binding, the Ad fiber protein interacts with the CAR to mediate cellular adhsion. Subsequently engagement of α_v integrins by the Ad penton base mediates internalization of the virion. After endosome disruption, the viral capsid transits to the nucleus for delivery of genetic material.

ward, an RGD-motif within the Ad penton base engages α_v integrins (119) on the cell surface to mediate internalization by clathrin-coated pit endocytosis (120, 121). Subsequently, acidification of the endosome exposes hydrophobic domains in the penton base that mediate endosomal escape into the cytoplasm (120-125). Finally, Ad particles transit to the nucleus along the cellular cytoskeleton, where it translocates into the nucleus and uncoats the viral genome (126). A breakthrough in the understanding of Ad biology, CAR was first identified by Bergelson et al. as the primary adenoviral receptor (118). This receptor, as the name might indicate, also facilitates entry of Coxsackievirus. Subsequently, Freimuth et al. demonstrated that, when generated as a recombinant protein, soluble CAR could indeed block Ad infection (127). More recently, expression of this receptor was found to correlate with Ad infection. Several studies have highlighted the concept that CAR deficiency is the single most common obstacle to gene transfer (128-132). To overcome a deficiency of native adenoviral receptors or alternately to direct an adenovirus specifically toward a target cell, strategies to target viral entry have been endeavored. Because it represents the means for initial virus to cell binding, the fiber protein has been at the center of efforts to target the Ad. Despite the theoretical simplicity of this approach, retargeting the adenovirus has proven to be a challenging feat. A large body of work surrounding the basic biology of the adenoviral fiber has suggested means to accomplish targeting. The base of the adenoviral fiber contains a nuclear localization signal (133) that directs the fiber protein to the location of capsid assembly and further engages with penton base proteins to create a penton complex. The shaft is a long triple-helical structure distal to the base, whose function is to extend the knob away from the capsid (134). Perhaps by extending the binding domain

away from the capsid, the virion can minimize steric hindrance around the binding region. The C-terminal knob domain is the functional domain of the fiber actually implicated in cellular binding. Specifically, it contains the CAR binding motif used for cell adsorption of the virion (135). The knob region also bears the signal responsible for trimerization of the three fiber monomers into a single fiber unit (136). Trimerization of the fiber is essential for interaction with the penton base, and further, insertion of the fiber into the base is requisite for proper assembly of the Ad capsid (137).

The first objective of modifications to the Ad fiber was the addition of targeting ligands to the fiber to allow specific binding to cells of interest. Early attempts sought to merely append ligand candidates as a C-terminal fusion with the existing fiber. Specifically, Michael et al. demonstrated that fiber prepared as a fusion protein would trimerize after addition of a 10 amino acid linker and 10 amino acid ligand (138). Subsequently, Wickham et al. extended these findings by illustrating that complete virions bearing poly-lysine or RGD-binding motifs on the C-termini of their fibers could enhance gene transfer relative to untargeted Ad (139). Nevertheless, the failure of chimeric fibers to trimerize with extensions as short as 27 amino acids suggested the trimerization domain was sensitive to conformational changes (136). The trimerization of this protein is essential to its ability to associate noncovalently with the Ad capsid (137), and thus this feature must be preserved when making modifications to the fiber.

In that specific gene delivery to a limited target population of cells is paramount, a second major obstacle includes the widespread tropism of the adenovirus (114). Thus, mere addition of a targeting ligand to the fiber without excision of this native binding function would still allow significant ectopic gene transfer. The inability to resolve

distinct cellular binding and fiber trimerization domains in the knob domain have precluded mere replacement of the native binding domain with a targeting motif without compromising the trimerization domain. Therefore, the goal of modifications to the Ad capsid is to eliminate the native binding capacity of the virion and replace it with a novel targeting motif, all the while maintaining the trimerization potential of the fiber. Recently, efforts have been initiated to replace the knob in its entirety with a heterologous trimerization domain that would be conducive to addition of targeting motifs. In the meantime, alternative strategies have been devised to establish basic concepts for retargeting of Ad vectors.

In their simplest embodiment, bispecific Abs are mAbs chemically conjugated to create a single entity with dual affinity for two distinct targets. Bispecific Abs had been previously employed for cell-specific targeting of both cytotoxic pharmacologic agents (140) and T cells (141). Subsequently, the use of bispecific Abs for targeting retroviral vectors was described. Through dual affinity for a viral coat protein and a target receptor, these entities could promote specific gene transfer to otherwise refractory cell types. In nature, where binding of the retrovirus mediates conformational changes in the fusion domain essential for cell fusion to occur, binding by a nonnative mechanism impaired the ability of retrovirus to enter the cell after binding (102). In this regard, viruses with distinct binding and cell fusion domains would, in theory, lend themselves more reliably to retargeting. For this reason, it was apparent that the Ad with its functionally distinct knob and penton base domains was particularly well suited for this strategy (119, 142). Toward the goal of a conjugate-targeted Ad vector, the work of Douglas et al. first illustrated the use of a Fab fragment of a neutralizing anti-Ad fiber antibody chemically

conjugated to the growth factor folate (143). Importantly, the neutralizing capacity of the anti-Ad antibody abolished native binding of the Ad capsid, thus allowing specific gene transfer only to cell types expressing the receptor of interest. Wickham et al. further extended the concept that basic steps of Ad entry could be reconstituted through modifications of the virion (144). Through Ab based targeting to the internalizing CD3 receptor T cells, the deficiency of the α_v integrins as an internalizing secondary receptor was overcome by targeting an alternate internalization pathway.

Racing Against the Clock: Combating Tansient Gene Expression

Perhaps foremost among drawbacks of Ad vectors is the limited duration of gene expression. This fleeting occupancy of Ad genome in the host cell stems primarily from immune-based clearance of Ad-infected cells (145, 146). In any case, the short-lived nature of Ad gene transfer is almost antithetical to the entire premise of gene therapy, where lifelong correction of a gene defect is often mandatory. Even so, this property is not entirely incompatible for an immunotherapy context, wherein Ad-transduced cells will likely have already transmitted a signal to other immune effector cells. All evidence would suggest that DC can activate T cells shortly after migration to lymphoid organs; thus, loss of gene expression after several weeks is insignificant. Naïve T cells become activated between 12 to 30 h of exposure to Ag-laden DC (147). Rendering immune clearance of DC even less significant, the duration of DC in nature is believed to be rather short-lived. Specifically, Ag pulsed DC disappear from draining lymph nodes by as early as 48 h (148). Foremost, DC have been shown to undergo apoptosis in vitro after engagement with Ag specific T cells; the role of Fas/FasL has been implicated in

this process (33). T cell-induced apoptosis of DC may serve as a regulatory mechanism to ensure only the most recently acquired Ags activate T cells. In any case, CTL eradication of Ad-transduced cells is believed to take much longer, between 7-14 days (149, 150). Much like a tandem marathon, once the signal has been passed from the Ad-infected cells to a T cell, its destiny has been fulfilled.

One of the primary setbacks of using Ad vectors as a gene therapy vector has been the anti-Ad immune response. Almost universal prior exposure by most of the population to Ad renders antiviral immunity a legitimate obstacle to gene therapy approaches that employ Ad vectors. Both humoral and cellular immune responses have been shown to reduce the effectiveness of Ad-based vector approaches (151). The Ad-neutralizing Abs that emerge in the humoral response block binding and entry of Ad, effectively compromising Ad entry and subsequent gene transfer. Cellular immunity, in contrast, is the primary mechanism by which cells that have already been infected are eradicated (145, 150). Current evidence suggests this response is directed at viral gene products as well as the transgene. The consequences of anti-Ad cellular immunity are evidenced by dramatic prolongation in the duration of gene expression either when T cell function is impaired by agents such as cyclophosphamide or in animals deficient in T cells (145, 150). These studies have highlighted the evidence that both E1a-independent background expression of viral gene products and expression of the transgene trigger cellular immunity through presentation in the context of MHC I on the surface of the host cell. Perhaps most compelling is the work by Jooss et al., who demonstrated that relative to a nonimmunogenic virus such as the adeno-associated virus, Ad initiates a powerful immune response directed at gene products of both the Ad and the transgene (151). Spe-

cifically, T cell responses directed at Ad and transgene gene products are a consequence of Ad infection of dendritic cells. Conversely, such immune responses are not observed after gene delivery by adeno-associated virus, a virus established not to infect DC. At first glance, this aspect would seem to negate the utility of DC that had been infected with Ad not only intentionally, but also with high efficiency. Conceivably, such DC would initiate or magnify existing Ad immunity in a manner that would eradicate the Ad-infected cells and thus compromise the effectiveness of Ad-modified DC.

With the current generation of Ad vectors, several features suggest that for immunotherapy contexts, the immune response to Ad is of less consequence for two reasons. These are the short timeframe for activation of T cells and the inherent suicidal tendency of DC after T cell engagement. To this end, Kaplan et al. have shown that mice preimmunized to Ad by nasal challenge are still susceptible to treatment by Ad-modified DC. As discussed previously, DC activation of T cells occurs in a relatively shorter timeframe, likely before the cells would be subject to immune clearance.

It is not unreasonable to expect that with each subsequent dose of infected cells, the memory immune response should become more rapid and robust, eventually neutralizing any further treatments. Regardless, as with conventional vaccinations, DC-based approaches would conceivably entail only a minimal number of treatments. Moreover, the abrogation of cellular immunity through elimination of viral gene expression through UV inactivation (145) suggests that a new generation of "gutless" Ad vectors with major deletions of viral genes may be less likely to instigate a cellular immune response (152). In any case, as with the transient gene expression profile of Ad, immune clearance of Ad-

infected DC becomes less significant once a signal has been relayed into a more enduring T cell response.

Human Papillomavirus E7: A Model For Tumor-Specific Ags

Viral Ags are particularly well suited for immunotherapy approaches because they are almost exclusively tumor specific. Their foreign nature imparts to them more immunostimulatory capacity than would be expected from a self protein. Examples of such pathogens include EBV, hepatitis B, and human papillomavirus (HPV), in the pathogenesis of lymphoma/nasopharyngeal carcinoma, hepatocellular carcinoma, and cervical cancer, respectively (17). Among these instigators, HPV Ags are especially attractive, stemming from the almost exclusive association of cervical cancer with HPV as well as the reliable expression of HPV Ags in these lesions.

Papillomaviruses are double-stranded DNA viruses. Most commonly known for their notorious reputation as the causative agent of anogenital warts, most types of HPV result in benign proliferations (153). Nonetheless, a subset of the papillomaviruses, the so-called "high-risk" HPV, such as types 16 (HPV16) and 18, have a tendency to progress to anogenital malignancy (153). Although infection by a high-risk HPV, first evidenced by genital warts, does not always proceed to cancer, the association of human cervical cancer with prior infection by HPV is upwards of 90% by several reports. Latency following infection is implemented by the cellular protein Ying-Yang-1 (YY1) (154). Cellular transformation by HPV centers around two of its gene products, E6 and E7. Specifically, the E6 and E7 gene products are known to compromise the function of the tumor-suppressor p53 and pRb gene products, respectively. It is by this mechanism

that HPV is able to transform cells in the presence of other precipitating factors. The tumor suppressor gene product p53 is instrumental in mediating G1 arrest following exposure to ionizing radiation or DNA damage (155). Through its association with p53, the HPV oncoprotein E6 targets p53 for degradation by ubiquitin-mediated proteolysis (156). In the absence of the p53 checkpoint, chromosomal abnormalities may ensue (157, 158). Likewise, the retinoblastoma tumor suppressor protein (pRb) is an important regulator of E2F transcription factor function. In a hypophosphorylated state, pRb sequesters E2F and effectively represses transcription of E2F-responsive genes (159). HPV16 E7 binds to the hypophosphorylated state of pRb, blocking its capacity to complex with E2F and culminating in dysregulation of the cell cycle (160, 161). Mutations in the pRb binding domain of E7 completely abolish soft agar growth in rodent fibroblasts or rodent primary cultures (162, 163). Also of note, the HPV gene product E2 is known to maintain regulation of E6 and E7 expression through repression of transcription of those genes (164).

Although the HPV genome can exist long term as an episome, in many cases of cervical carcinomas, the papillomavirus has become integrated into the host genome (164). Although the site of integration is random and incomplete, it is clear that selective preservation of certain viral fragment plays a profound role in viral gene expression in these cells. Specifically, integration of HPV can promote dysregulation of E6 and E7 by several mechanisms. Foremost, the E2 gene is absent or disrupted in integrated viral sequences; liberation from E2 repression facilitates transcription of E6 and E7 genes (164). Additionally, deletion of the YY1 binding site during integration relieves latency-based suppression of the E6 and E7 genes (165). Finally, integration eliminates control

sequences that otherwise decrease mRNA stability, further deregulating expression of these oncogenes. In light of these findings, it should come as no surprise that in culture, cervical cancer cells with integrated viral sequences rapidly overgrow cells containing episomal HPV genomes (166). In and of themselves, however, these events do not necessarily result in cancer unless subsequent somatic mutations ensue (153), as by the expression of a second oncogene, such as ras or fos (162).

The immune system plays a crucial role in the control of cervical cancer, as evidenced by spontaneous resolution of HPV lesions (167) and by an increased number of HPV-infected individuals developing this cancer during immunosuppression (48, 49). The fact that HPV-induced neoplasms can be controlled by the intact immune system suggests that an immunotherapy approach might be of utility in resolving these lesions. Many tumor-associated Ags, EGFR or gp100 of melanoma for instance, are merely overexpressed on cancerous cells; thus, normal tissues might fall under immune assault during therapy against these markers (14). Since the genes of HPV are not expressed by normal cells, the use of their gene products as targets represents a way to accurately distinguish normal from cancer cells while minimizing the potential for autoimmunity. Moreover, the continued expression of E6 and E7 is known to be essential for maintenance of the transformed phenotype (168). By the time a cell has reached transformation, usually only the E6 and E7 regions are reliably expressed in these cells (164). Thus, the list of potential candidates for vaccination against HPV is certain to include at least one of these Ags.

In fact, a great deal is known about the existence of APC in the reproductive tract, in particular as relates to cervical lesions caused by HPV. Foremost, a significant deple-

tion of Langerhans cells (LC) has been associated with cervical condylomas (169-174). It has been suggested that such a deficiency in afferent immunity might predispose a person to chronic HPV infection and subsequent progression of HPV-induced transformation (172, 174). Relevant to the DC microenvironment, HPV immortalization causes a decrease in cytokines important in DC function, such as IL-1β and GM-CSF (175). Moreover, IL-10, a potent DC-inhibitory cytokine is known to be expressed in malignant lesions caused by HPV (169). If the presumed role of cervical LC as an important deterrent to malignant progression is correct, then the finding that smoking has been linked to a significantly reduced number of cervix DC (170) would explain a well-established correlation of smoking as a coprecipitating factor of cervical cancer. These factors suggest that absence or dysfunction of DC may play a significant role in the progression of HPV-induced lesions.

Experimental Design

The objective of the detailed herein was to establish the means to genetically modify DC and to evaluate the utility of these cells in a vaccination context. Regardless of the adjuvant or the formulation of the vaccine preparation, conventional vaccines are ultimately dependent upon APC to collect Ag from the site of vaccination. Evidence suggests that in some instances, the uptake of Ag from the surroundings and proper Ag presentation may serve as a limiting factor for initiation of an immune response (17, 21, 176). Subsequently, it was established that by delivery of an Ag encoding gene directly into DC, a renewable source of intracellular Ag could be created that would entirely bypass native Ag acquisition.

A number of strategies, albeit inefficient, have been described for genetic modification of DC (176, 177). Despite demonstrating the effectiveness of DC-based therapies, the practicality of these approaches is limited to small-scale vaccinations. Simplification of this procedure will ultimately become necessary if genetically modified DC are to be employed as a routine clinical therapeutic agent. As with other gene transfer modalities, the delivery of Ag-encoding genes by Ad vectors is likewise inefficient. In other instances of cells that are resilient to Ad gene transfer, the basis has been attributed to a deficiency of Ad entry receptors (128-132). The first manuscript here defines the hurdles to gene transfer in human monocyte-derived DC and describes a means by which to overcome these obstacles. In identifying the key defect as an absence of viral binding receptor, we have employed bispecific Abs to redirect Ad binding to an alternate binding receptor, in particular, targeting Ad to CD40 initiated functional changes in the DC commensurate with its role in DC maturation. Nevertheless, the non-specific activation that is characteristic of an allo-mixed leukocyte reaction left unresolved whether this strategy could elicit immunity in an Ag specific manner and whether this phenomenon would improve DC vaccinations in an in vivo setting.

In this regard, the second manuscript describes the use of this system in a murine model. An animal model is particularly well suited for establishing the baseline in vivo efficacy of this approach. In particular, I chose a model of human cervical cancer, both because of the high association of cervical cancer with HPV and because of the exquisitely tumor-specific nature of the HPV E7 tumor Ag. Using this system, it was possible to compare DC modified by targeted and untargeted Ad vectors in both a prophylactic and therapeutic setting. Moreover, Ag specificity could be illustrated either from the

viewpoint of a viral vector carrying the gene for an irrelevant Ag or E7-based vaccination against a tumor negative for expression of the target Ag. We opted to use an intradermal mode of vaccination both because the skin is a native point of egress for DC of the skin, LC (14), and because DC tracking studies have indicated more effective migration to secondary lymphoid organs after intradermal injection (178).

A consequence of immune clearance of viral-infected cells is that the long-term gene expression is compromised for Ad vectors, as illustrated in several studies (145, 146). This shortcoming has emerged as a significant obstacle for most gene therapy applications. The dissimilar objective in vaccination approaches, however, suggests this feature might not necessarily be incompatible with these applications. In fact, the naturally short lifespan of DC suggests that even a brief period of genetic modification may have lasting effects on immunity. Much as a brief encounter with a pathogen can result in long-term immunity to subsequent infection, so too genetically modified DC may provide enduring immune modulation, even after the instigating DC have been destroyed. To this end, we have also examined the potential of genetically modified DC to instigate an antitumoral immune response following prevaccination of animals with Ad-infected DC.

MATURATION OF DENDRITIC CELLS ACCOMPANIES HIGH EFFICIENCY GENE TRANSFER BY A CD40-TARGETED ADENOVIRAL VECTOR

by

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Abstract

Important therapeutic applications of genetically modified dendritic cells (DC) have been proposed; however, current vector systems have demonstrated only limited gene delivery efficacy to this cell type. By means of bispecific Abs we have dramatically enhanced gene transfer to monocyte derived DC (MDDC) by retargeting adenoviral (Ad) vectors to a marker expressed on DC, CD40. Ad targeted to CD40 demonstrated dramatic improvements in gene transfer relative to untargeted adenoviral vectors. Fundamental to the novelty of this system is the capacity of the vector itself to modulate the immunological status of the MDDC. This vector induces DC maturation as demonstrated phenotypically by increased expression of CD83, MHC, and costimulatory molecules, as well as functionally by production of IL-12 and an enhanced allostimulatory capacity in a MLR. In comparing this vector to other Ad-based gene transfer systems, we have illustrated that the features of DC maturation are not a function of the Ad particle, but rather a consequence of targeting to the CD40 marker. This vector approach may thus mediate not only high-efficiency gene delivery but also serve a proactive role in DC activation that could ultimately strengthen the utility of this vector for immunotherapy strategies.

Introduction

A growing body of evidence suggests that dendritic cells (DC) play a pivotal role in the immune system (1-4). Foremost, DC are recognized to serve as central mediators

of T cell-based immunity. Stemming from this key function, DC have been proposed for use in a number of clinical immunotherapy strategies. In this context, it has become clear that genetic modification of these cells can promote immunity against pathogenic entities, both infectious and tumorigenic (5-8). Importantly, all of these strategies are predicated upon efficient vectors for gene delivery to DC. A number of approaches have been investigated for this purpose, albeit generally with poor efficiency of gene delivery (9, 10). One candidate explored for this application has been the replication-defective adenoviral (Ad) vector (9, 11). This vector has been suggested to be well suited for DC-based clinical applications by virtue of its high titer vector production and exuberant gene expression.

In spite of these theoretical advantages, the relative resistance of DC to Ad vectors has confounded the realization of gene-based immunotherapy strategies (9, 11, 12). We hypothesized that DC resistance to Ad-mediated gene transfer may stem from a paucity of the cellular receptors that mediate Ad entry. In permissive cells, the projecting Ad fiber-knob protein mediates binding to the Coxsackie-adenovirus receptor (CAR) on the cell surface, followed by internalization of the virion facilitated by interaction of Ad penton base with either of the α_v integrins $\alpha_v\beta_3$ or $\alpha_v\beta_5$ (13-15).

We have determined that while monocyte-derived DC (MDDC) exhibit adequate levels of the α_v integrins, they do not express CAR. We and others have previously shown the utility of redirecting Ad binding to alternate cellular receptors so as to mediate enhanced gene transfer (16-18). The CD40 receptor has been reported to play an essen-

tial role in promoting both DC activation as well as Ag presenting function. We reasoned that by targeting Ad to CD40, this vector approach might simultaneously mediate changes that would augment the immunostimulatory function of genetically modified DC. Here, we report that an Ad targeted to CD40 mediates both high-efficiency gene transfer as well as phenotypic and functional maturation of MDDC. We propose that such a vector may have utility in DC-based vaccination strategies.

Methods

Culture of MDDC. PBMC were isolated from heparinized peripheral blood of normal human volunteer donors by density centrifugation over Lymphoprep (Nycomed AS, Oslo, Norway). These cells were cryopreserved until use in RPMI 1640 medium supplemented with 12.5% dimethyl sulfoxide and 25% FCS (19). PBMC were suspended at a concentration of 3-5 million cells per ml in IMDM containing 50 U/mL penicillin-streptomycin, 1.6 mM L-glutamine, 0.01 mM 2–ME (complete medium), and 10% FCS and were allowed to adhere to the bottom of plastic culture flasks (NUNC, Intermed, Denmark) at 37°C. The adherent cells were cultured for an additional a further 6 days in medium supplemented with 1000 U/ml rIL-4 (Centraal Laboratorium van de Bloedtransfusiedienst, Amsterdam, The Netherlands) and 100 ng/mL GM-CSF (Schering-Plough, Madison, N.J.). Adherent MDDC released by 0.5 mM EDTA were pooled with nonadherent MDDC. These cells were characterized by presence of CD1a and CD11c expression as well as the absence of lineage markers, such as CD3, CD14, CD19

and CD56, by FACS. These cells also demonstrated typical DC cell morphology such as dendritic processes and clustered cells.

Viruses and cell lines. AdCMV Luciferase (AdCMVLuc), a first generation E1-, E3-deleted vector expressing firefly luciferase from the CMV immediate early promoter, was obtained from Robert Gerard (University of Leuven, Leuven, Belgium). Ad green fluorescent protein (AdGFP) was obtained from Corey Goldman (University of Alabama at Birmingham, Birmingham, Alabama).

Viruses were propagated and plaque-titered on the 293 Ad propagation line and purified by double centrifugation on CsCl gradients. All virus aliquots were stored at -80° C until use. The neutralizing murine mAb 1D6.14 specific for the carboxy-terminal, receptor-binding knob domain of Ad serotype 5 fiber has been previously described (17). The pharyngeal epithelial line KB was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and was maintained in DMEM with 4.5 g/L glucose.

Abs and conjugates. The anti-CAR mAb RmcB (a generous gift from Robert W. Finberg, Harvard Medical School, Boston, MA) has been previously described (13). Murine mAb LM609 to $\alpha_v\beta_3$ integrin and P1F6 to $\alpha_v\beta_5$ integrin were purchased from Chemicon (Temecula, CA) and Life Technologies (Gaithersburg, MD), respectively. For maturational and lineage marker analysis, Abs used were directly conjugated to FITC or PE. These included HB15a (anti-CD83), MAB89 (anti-CD40) (Immunotech,

Marseille, France), L243 (anti-HLA-DR) (Becton Dickinson, San Jose, CA), 2331 (anti-CD86), HA58 (anti-CD54), and TU169 (anti-HLA-DQ) (Pharmingen, San Diego, CA). The hybridomas G28.5, TS2/16.2.1, and 528, producing mAbs against CD40, the β1 integrins, and epidermal growth factor receptor (EGFR), respectively, were purchased from ATCC. These hybridomas were used to generate ascites in SCID mice. Abs were purified on a fast liquid protein chromatography system using HiTrap protein A columns (Pharmacia, Piscataway, NJ) and the mAb purification system binding buffer system (Bio-Rad, Hercules, CA). The 1D6.14 mAb was digested with immobilized papain (Pierce, Rockford, IL), and Fab fragments were purified by negative selection of Fc fragments using the HiTrap Protein A columns. Bispecific Abs consisting of the 1D6.14-neutralizing anti-Ad knob Fab fragment and one of several anti-receptor antibodies (G28.5, TS2/16.2.1, or 528) were prepared by chemical cross-linking with *N*-succinimidyl 3-(2-pyridldithio) propionate (SPDP), as previously described (20). Conjugates using the G28.5 mAb, TS2/16.2.1 mAb, and 528 mAb are henceforth designated as Fab-anti-CD40, Fab-anti-β1-integrins, and Fab-anti-EGFR, respectively. Lipofectamine (Life Technologies) was used for liposome-complexed Ad, as previously described (11).

Conjugate titration to determine optimal conjugate to virus ratio. To determine the amount of retargeting conjugate necessary to optimally enhance gene transfer, the conjugate was titrated with AdCMVLuc at a multiplicity of infection (MOI) of 100

and used to infect MDDC by previously described methods (17). The mass of conjugate corresponding to the highest levels of luciferase gene expression was termed an "optimal ratio of conjugate to virus" and was used in all subsequent experiments. This ratio was determined to be 30.3 ng:2.4 X 10^6 PFU for the Fab-anti-CD40 and Fab-anti-β1 integrins conjugates and 60 ng:2.4 X 10^6 PFU for the Fab-anti-EGFR conjugate. Adenovirus conjugated with the optimal ratio of Fab-anti-CD40, Fab-anti-β1 integrins, and Fab-anti-EGFR will be referred to henceforth as CD40-targeted Ad, β1-integrin-targeted Ad, and EGFR-targeted Ad, respectively.

AdCMVLuc infection and luciferase analysis. Twenty-four thousand MDDC in a volume of 50 μl of complete RPMI with 2.5% FCS were distributed to individual microcentrifuge tubes in triplicate for each test condition. The use of microcentrifuge tubes enabled simplified infection and washing of the pooled adherent and nonadherent MDDC. Cells were pre-blocked for 30 min as indicated with either media or blocking agent consisting of the unconjugated anti-CD40 antibody at 100 μg/ml. All blocking was performed at 4°C to minimize premature receptor modulation from the cell surface. Conjugate and virus were incubated for 30 minutes at room temperature in a volume of 20 μl per each test condition. Following incubation, the mixture was diluted such that 100 μl was used to infect each microcentrifuge tube or well of cells. The amount of virus in this volume corresponded to an MOI of 100. Following further incubation for a period of 30 min at 4°C, cells were washed, resuspended in complete RPMI with 10%

FCS, and plated on polylysine-coated 24-well plates (Becton Dickinson) before transition to 37°C. Following 24 hours of incubation postinfection, wells were processed using the Promega (Madison, WI) luciferase assay kit. The lysates were evaluated on a Lumat luminometer (Wallac, Gaithersburg, MD). The results were normalized for the number of cells present during infection.

Analysis of differential MOI between CD40-targeted and untargeted Ad. Fab-anti-CD40 was complexed with AdCMVLuc at a concentration corresponding to 1000 MOI. Subsequently, this mixture was serially diluted to MOIs of 100, 10, and 1. Simultaneously, samples of the same MOIs of Ad but without retargeting conjugate were prepared. MDDC were then infected and processed as described under "AdCMVLuc Infection and Luciferase Analysis" methods.

Flow cytometry analyses. For analyses of Ad entry receptors, MDDC or KB were stained using mAb with affinity for CAR, $\alpha_v\beta_3$, or $\alpha_v\beta_5$, followed by a FITC-labeled rabbit anti-mouse Fc-specific secondary antibody (Jackson Immunoresearch Labs, West Grove, PA). These samples were analyzed on a FACStar using Cellquest FACS analysis software (Becton Dickinson). To assess changes in expression of maturation and lineage markers, MDDC were batch-infected using AdCMVLuc complexed to media (untargeted Ad), lipofectamine (liposome-complexed Ad), or the optimal ratio of the indicated Fab-anti-receptor conjugates. Twenty-four hours postinfection, cell staining and FACS

analysis were performed using mAbs with affinity to CD83, CD40, HLA-DR, CD86, CD54, or HLA-DQ directly conjugated to FITC or to PE. Samples were assessed by FACS analysis. To determine the percentage of MDDC transduced, 24 h before to analysis, cells were infected with AdGFP that had been complexed for 30 min with Fab-anti-CD40, Fab-anti-β1 integrins, or liposomes. After incubation for 1 h at 37°C, cells were washed with PBS and left to incubate in microcentrifuge tubes in complete RPMI with 10% FCS for the duration of the 24 h incubation.

DC functional assays. For allogeneic MLR, MDDC were infected as described in *Materials and Methods*. Seventy-two hours after infection, these DC were added as stimulator cells to round-bottom 96-well culture plates (Nunclon Delta, Intermed, Denmark) at graded doses reflecting the indicated responder-stimulator ratios (R:S). Nonadherent lymphocyte fractions were used as a source for responder cells, 100,000 lymphocytes were added per well to the allogeneic MDDC. The cells were cultured for 3 days in complete medium with 10% human pooled serum (CLB, Amsterdam, The Netherlands). During the last 18 h, [^{3}H]thymidine was added (0.4 μCi per well) (Amersham, Aylesbury, U.K.), after which the cells were harvested onto fiberglass filters and [^{3}H]thymidine incorporation was determined using a flatbed liquid scintillation counter (Wallac). IL-12 production was assessed using an ELISA as described (21). Forty-eight hours post-infection, assessment was made of supernatants in which a million cells had been incubated in 1 mL of media.

Results

MDDC are deficient in CAR. Using Abs with affinity for CAR or specific for each of the integrin heterodimers $\alpha_v\beta_3$ and $\alpha_v\beta_5$, we assessed MDDC as compared with an Ad-permissive epithelial line, KB. This analysis revealed an absence of CAR on DC but confirmed the expression of $\alpha_v\beta_5$ (Fig. 1*A*) and $\alpha_v\beta_3$ (data not shown). This contrasts to KB cells, which express both CAR and $\alpha_v\beta_5$ (Fig. 1*B*). Based on understanding of the Ad entry pathway (13-15), these findings suggest that the absence of CAR expression is the likely cause for the resistance of DC to adenovirus.

Enhanced, CD40-specific gene transfer by CD40-targeted Ad. Based on our previous success at mediating CAR-independent gene transfer using retargeted Ad, we postulated that a similar strategy targeting CD40, a marker expressed on DC, might enable enhanced gene transfer in spite of the absence of CAR expression. For this purpose, a bispecific Ab was generated through chemical conjugation of a Fab fragment of a neutralizing anti-fiber-knob mAb to a mAb with affinity for CD40, a receptor expressed on DC. MDDC were prepared by culture of adherent monocytes in the presence of IL-4 and GM-CSF, and the DC phenotype was confirmed by characteristic receptor expression and morphology. The Fab-anti-CD40 conjugate was titered against Ad to ascertain the optimal ratio of conjugate to virus as measured by improvements in gene transfer to MDDC (data not shown). The magnitude of gene expression mediated by Ad carrying the gene for luciferase, as well as specificity for CD40, was illustrated on MDDC and also on a

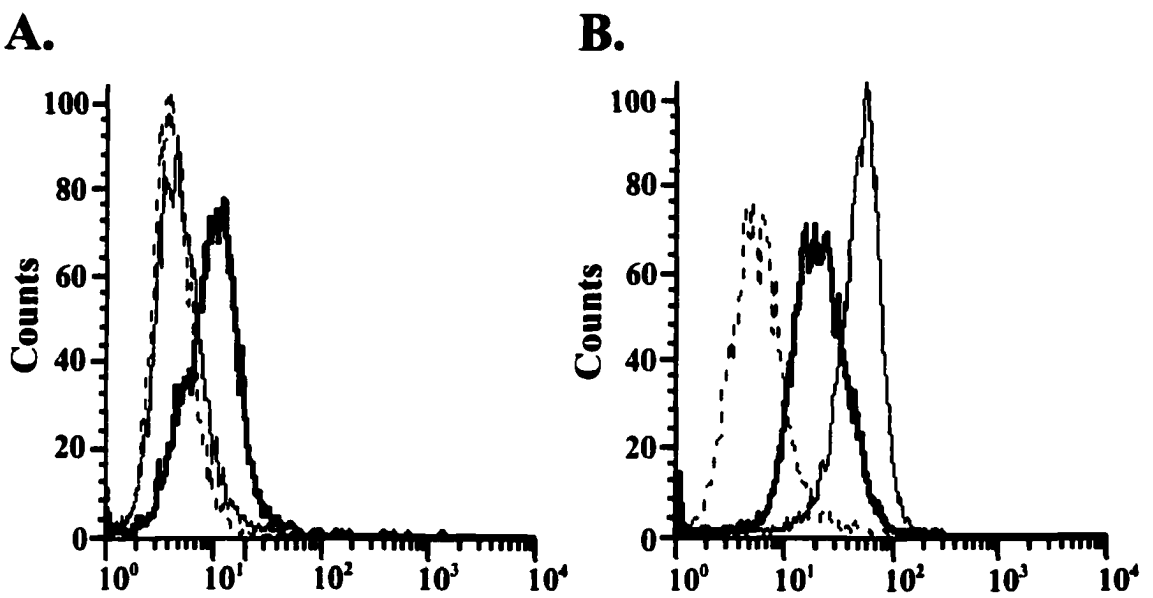

FIGURE 1. MDDC are deficient in the primary Ad-entry receptor. Relative to an isotype control (dashed line), MDDC (*A*) are negative for expression of CAR (thin line) but do express sufficient $\alpha_v\beta_5$ (thick line) as determined by flow cytometry, while the Ad permissive epithelial line KB (*B*) is positive for both of these receptors.

CD40-negative glioma line, D65, which like MDDC is both negative for expression of CAR and has been reported to be resistant to gene transfer by Ad (22). As illustrated in Fig. 2, compared with untargeted Ad, Ad complexed with Fab-anti- CD40 conjugate (CD40-targeted Ad) mediated several orders of magnitude enhancement in gene expression in MDDC. Furthermore, this enhancement was blocked by ~90% when cells were pretreated with an excess of the unconjugated anti-CD40 mAb. As evidence that the anti-CD40 mAb did not promote gene transfer by a mechanism independent of its association with the virion, no enhancement was observed in cells pretreated with unconjugated anti-CD40 mAb before infection with untargeted Ad. Further specificity was illustrated by failure of the Fab-anti-CD40 conjugate to enhance Ad-based gene transfer to the CD40-negative glioma, D65 (data not shown). The successful anti-CD40 blockade of enhanced gene transfer to MDDC, as well as the restriction of enhancements in gene transfer to cells expressing CD40, indicate that this vector is specifically targeted to cells by means of its affinity for CD40.

CD40-targeted Ad reduces the viral dose for a given magnitude of gene expression. Dose-related cytotoxic effects of adenoviral vectors can compromise potential applications (22). Thus, the capacity to reduce viral dose would increase the utility of adenoviral vectors immensely. To compare the efficacy of this retargeting strategy in MDDCs, CD40-targeted Ad was compared with untargeted Ad at several MOI (Fig. 3). The results illustrated that, with increased numbers of infectious particles per cell, gene

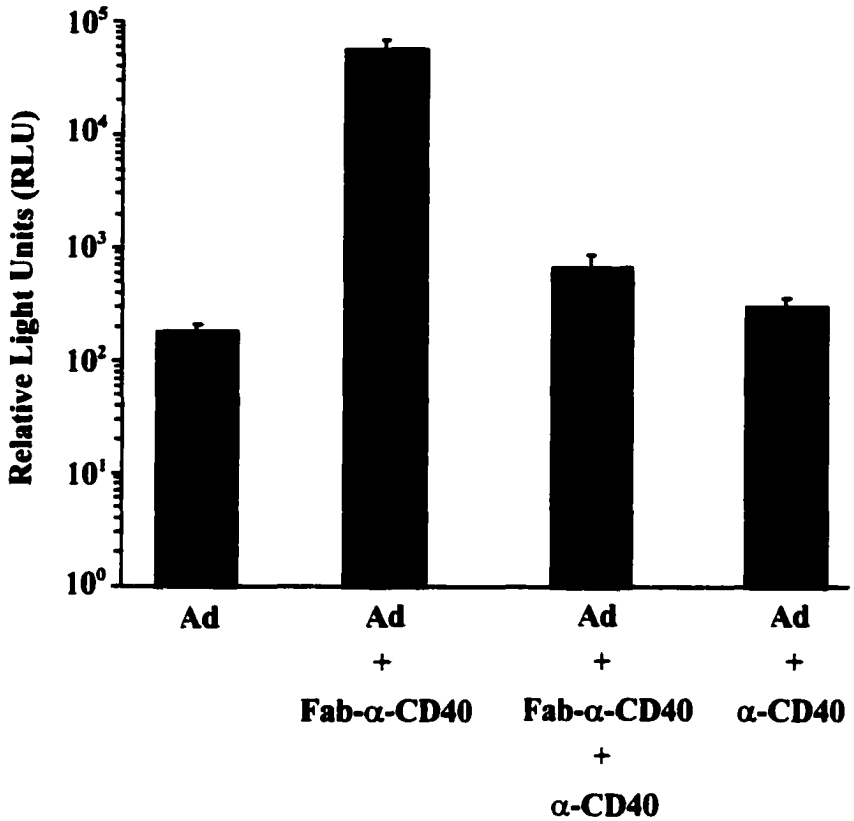

FIGURE 2. Ad targeted by Fab-anti-CD40 mediates enhanced magnitude of gene transfer that is specific for CD40. MDDC preincubated in either the presence or absence of unconjugated anti-CD40 mAb were infected with AdCMVLuc (MOI 100) either alone or complexed with Fab-anti-CD40. After 24 h of incubation, cells were assessed for expression of luciferase, measured as relative light units (RLU). The background of the luminometer is ~100 RLU in the absence of sample.

transfer was progressively increased under both conditions. Nevertheless, it is apparent that CD40-targeted Ad mediates a level of expression greater than untargeted Ad, even when 100-fold more virus is used. For example, CD40-targeted Ad attained a magnitude of gene expression at an MOI of 10 equivalent to that observed with untargeted Ad at an MOI of 1000. These findings indicate that by adenoviral targeting to CD40, the number of infectious particles required to attain a given level of gene expression is reduced appreciably.

CD40-targeted Ad enables gene transfer to increased numbers of cells.

While luciferase gene transfer illustrated an overall increase in gene expression by CD40-targeted Ad, this assay can indicate only the magnitude of gene expression. To exclude the possibility that the enhancements merely reflect a small subset of DC that was transduced multiple times, we assayed the actual percent of cells transduced by Ad-mediated delivery of the gene for GFP. At this stage, we also employed two other high-efficiency Ad-based vector systems for comparison with CD40-targeted Ad. The Fab-anti-β_1 integrin conjugate has a similar construction to the CD40-targeting conjugate but with affinity for the widely expressed β_1 integrin subunit. Meanwhile, Ad complexed with liposomes has been described previously for gene transfer to MDDC (11). As an additional control, a conjugate (Fab-anti-EGFR) targeted to a receptor absent from MDDC, EGFR, was included to ascertain any nonspecific binding by conjugate-based strategies. GFP expression was quantitated through use of flow cytometry and indicated that Ad vectors

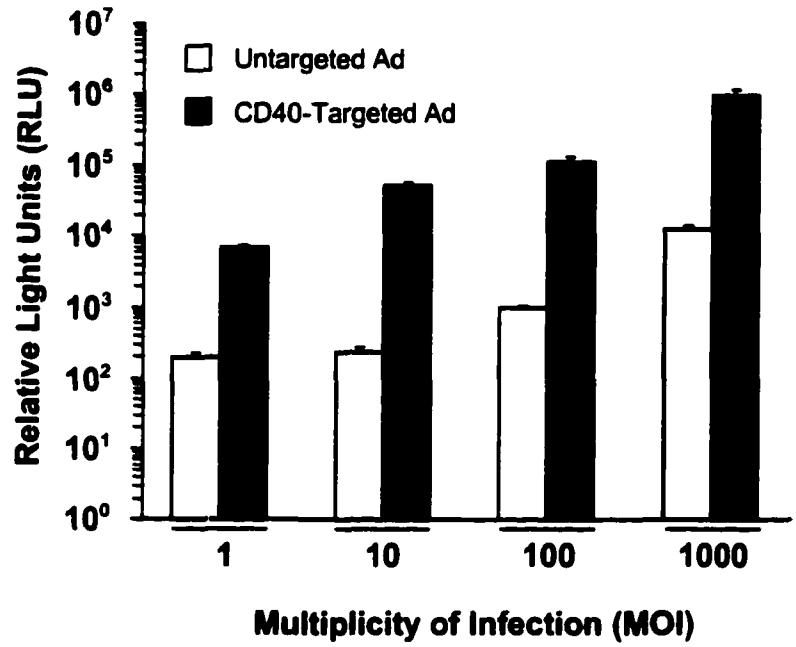

FIGURE 3. Targeting of Ad to CD40 reduces the viral MOI necessary to attain a given level of gene expression. Virus, either in the presence or absence of Fab-anti-CD40 conjugate, was incubated for 30 min and subsequently serially diluted to correspond to MOI of 1000, 100, 10, and 1. MDDC were infected for 1 h, and cells were assayed at 24 h for luciferase expression. The background of the luminometer is ~100 RLU in the absence of sample.

targeting to EGFR to enhance gene transfer illustrates the specificity of conjugate-based targeting. In aggregate, it is apparent that the CD40-targeted, β_1 integrin-targeted, and liposome complexed Ad-based vector systems mediate comparable levels of enhancement in gene transfer efficacy.

CD40-targeted Ad induces phenotypic and functional characteristics of mature DC. The anti-CD40 mAb employed in the targeting conjugate, G28.5, has been previously described for its cross-linking-dependent partial agonist activity on CD40 in B-cells (24). As activation of CD40 has been established to have profound effects on DC (3, 25, 26), we sought to evaluate the effect of CD40-targeted Ad on DC phenotype and function. To determine the effects on DC maturation by the retargeted Ad vectors or the CD40-retargeting conjugate alone, several markers were analyzed using flow cytometry. Cells infected 24 h previously were analyzed for expression of CD83, ICAM-1, CD86, HLA-DR (Fig. 5), as well as HLA-DQ (data not shown). While no alterations in DC phenotype were observed when Ad was used alone or when targeted by an irrelevant conjugate, Fab-anti-EGFR (data not shown), clear changes including augmented expression of CD86, HLA-DR, and HLA-DQ were observed with all three high-efficiency Ad gene delivery systems, as well as by the Fab-anti-β1 integrin-targeting conjugate alone (data not shown). Unique characteristics imparted by treatment with either Fab-anti-CD40 conjugate or CD40-targeted Ad included increased expression of CD83 and ICAM-1, features seen only negligibly, if at all, in cells infected with β_1 integrin-targeted

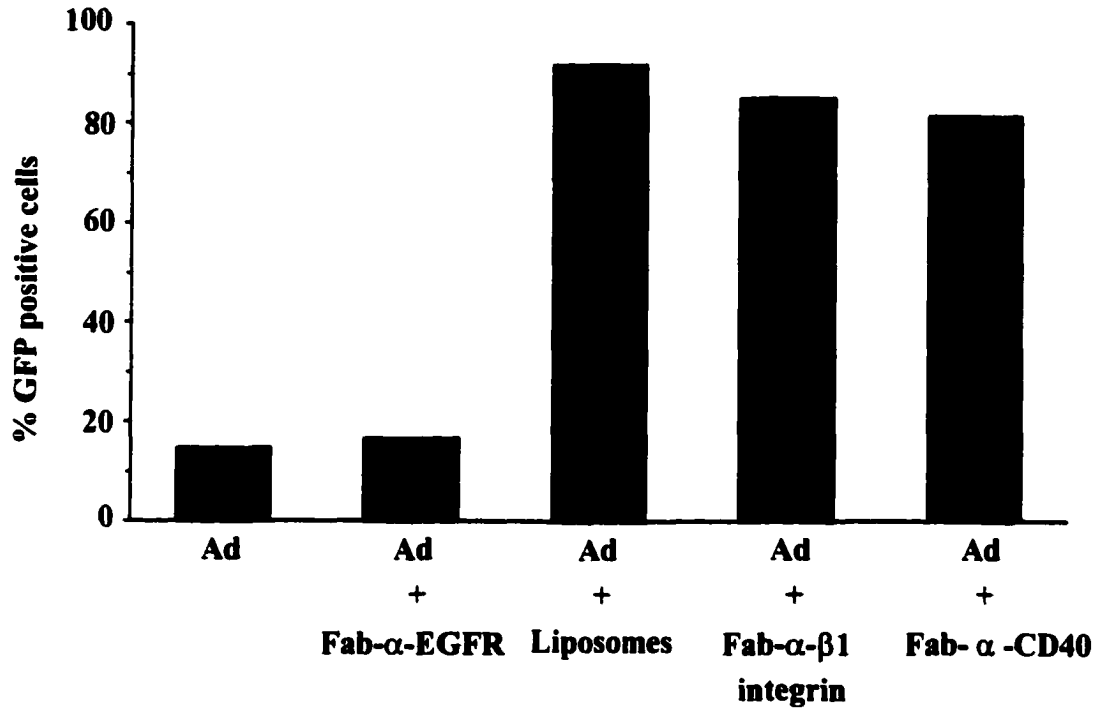

FIGURE 4. CD40-targeted, β_1 integrin-targeted and liposome-complexed Ad mediate comparable gene transfer to MDDC. MDDC were infected with Ad (MOI 100)-encoding GFP preincubated with one of the following: PBS, Fab-anti-CD40, Fab-anti-β_1 integrin conjugate, Fab-anti-EGFR conjugate or liposomes. After 24 h of incubation, the conditions were assessed using flow cytometry for expression of GFP and are displayed as percent GFP-positive cells based on analysis of 10,000 cells. This depicts a representative of two experiments.

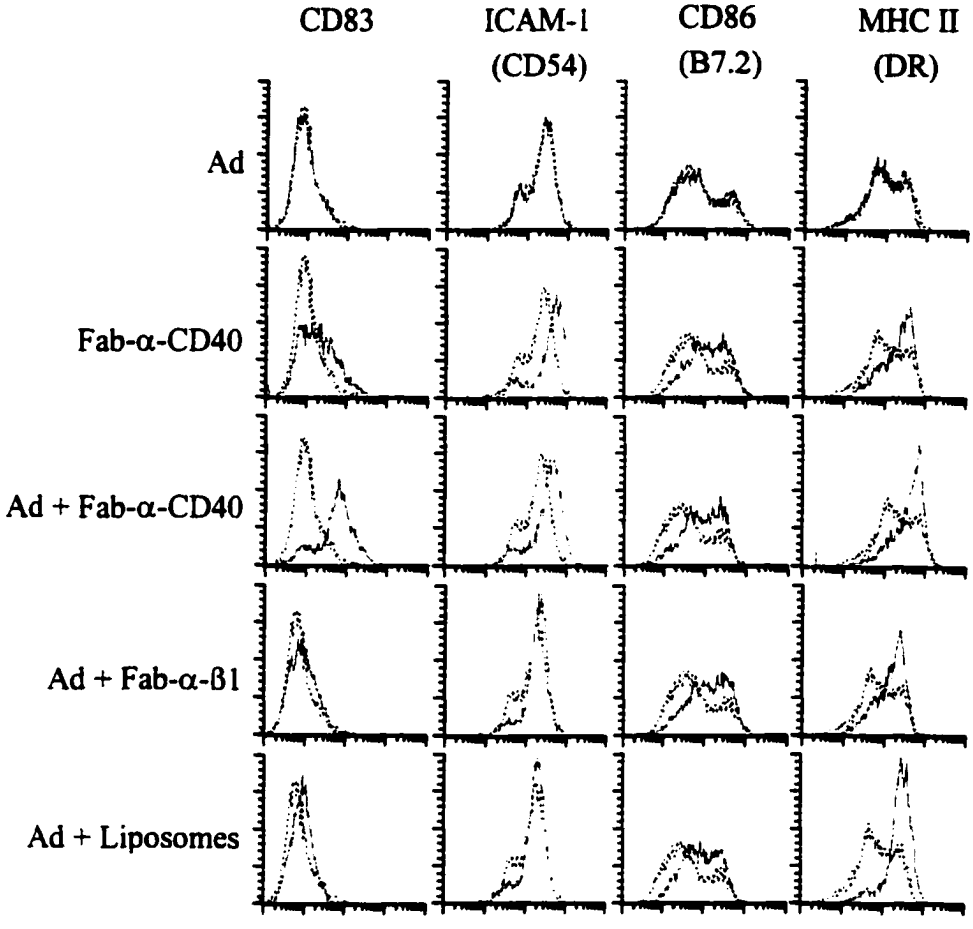

FIGURE 5. CD40 targeting induces expression of DC maturational markers. MDDC were treated as indicated and incubated for 24 h before anaiysis. Samples shown indicate expression of CD83, CD54, CD86, and HLA-DR (solid line) by flow cytometry and are depicted as compared with mock-transduced MDDC (dotted line). Abs directly conjugated to fluorophores were used for these analyses. An MOI of 100 was used in instances where Ad was employed. A total of 5,000 events per condition were counted.

or liposome-complexed Ad. Of note, CD83 enhancement appeared more remarkable with CD40-targeted Ad than with CD40-targeting conjugate alone. As an indication of functional maturation, MDDC-treated, using the targeted Ad vectors or Fab-anti-CD40 conjugate alone, were combined with responder cells from an allogeneic donor and tested for their capacity to elicit an MLR (Fig. 6). While Ad alone did not mediate any enhancement in MLR, use of Fab-anti-CD40 conjugate either in the presence or absence of Ad was able to promote enhanced MDDC reactivity in the allo-MLR relative to uninfected cells by an average of 2-fold ± 0.6 ($n = 4$). Moreover, the effect of conjugate alone was comparable to that seen with the conjugate plus virus. One possible explanation of the maturational effects observed with CD40-targeting could have been a viral-mediated effect from high-efficiency entry of Ad particles into DC. It was for this reason that DC were infected with the alternate high-efficiency Ad vectors β_1-integrin-targeted Ad or liposome-complexed Ad and tested in an MLR. The failure of these latter two vector systems to mediate notable enhancements suggests the maturation phenomenon is CD40-associated. As further evidence of functional maturation, MDDC supernatants were tested at 48 h postinfection for production of IL-12 (Fig. 7), a cytokine for which expression is characteristic of DC maturation and which plays a critical role in the induction of cellular immunity (12, 27). The results indicated that IL-12 levels were elevated in supernatants of cells treated either with Fab-anti-CD40 retargeting conjugate alone or with CD40-targeted Ad. In contrast, IL-12 augmentation was not observed for untargeted, EGFR-targeted, β_1-integrin-targeted, or liposome-complexed Ad.

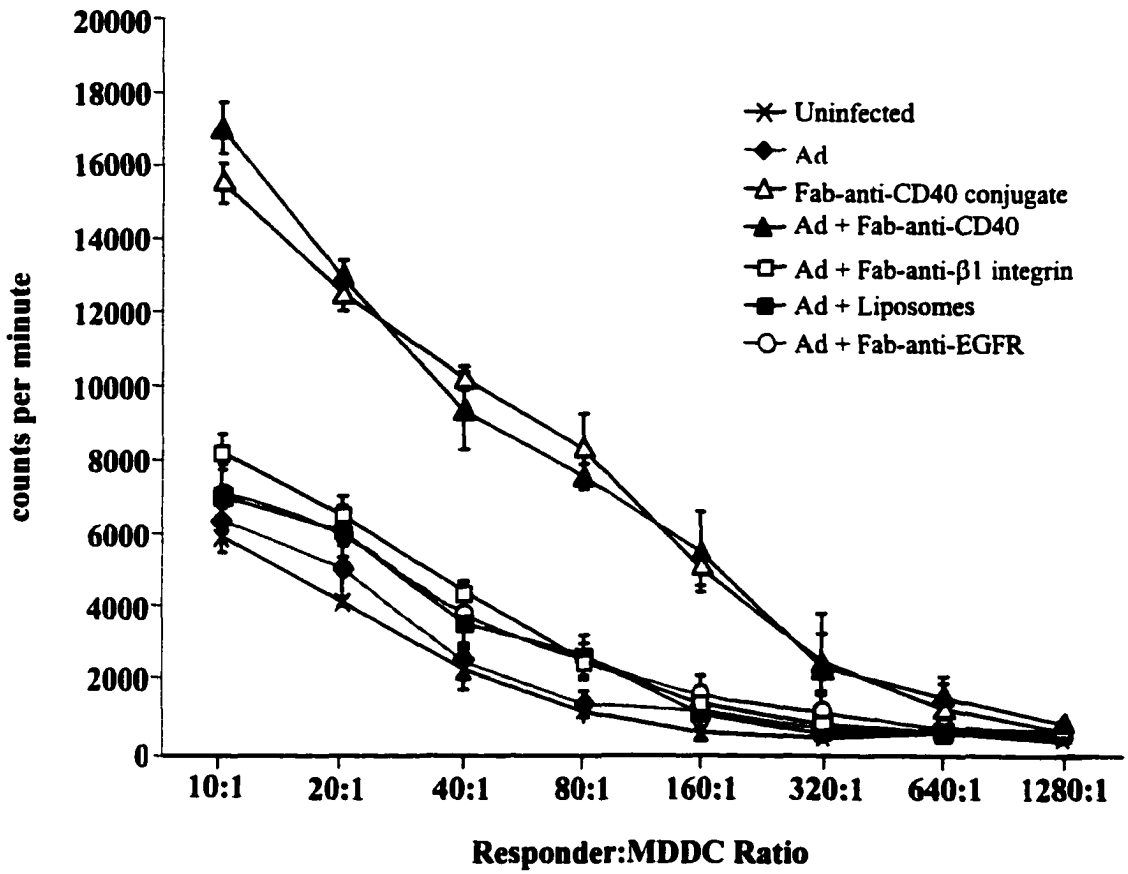

FIGURE 6. Ad targeting to CD40 mediates enhancement in the capacity to generate an allo-MLR. MDDC were infected (MOI 100) with the indicated conditions and, after 72 h of incubation, were mixed with nonadherent lymphocyte responder cells at the indicated responder-MDDC ratios. Cells were ^{3}H-labeled and assessed for cell-associated cpm after 3 days. This is a representative result from four experiments.

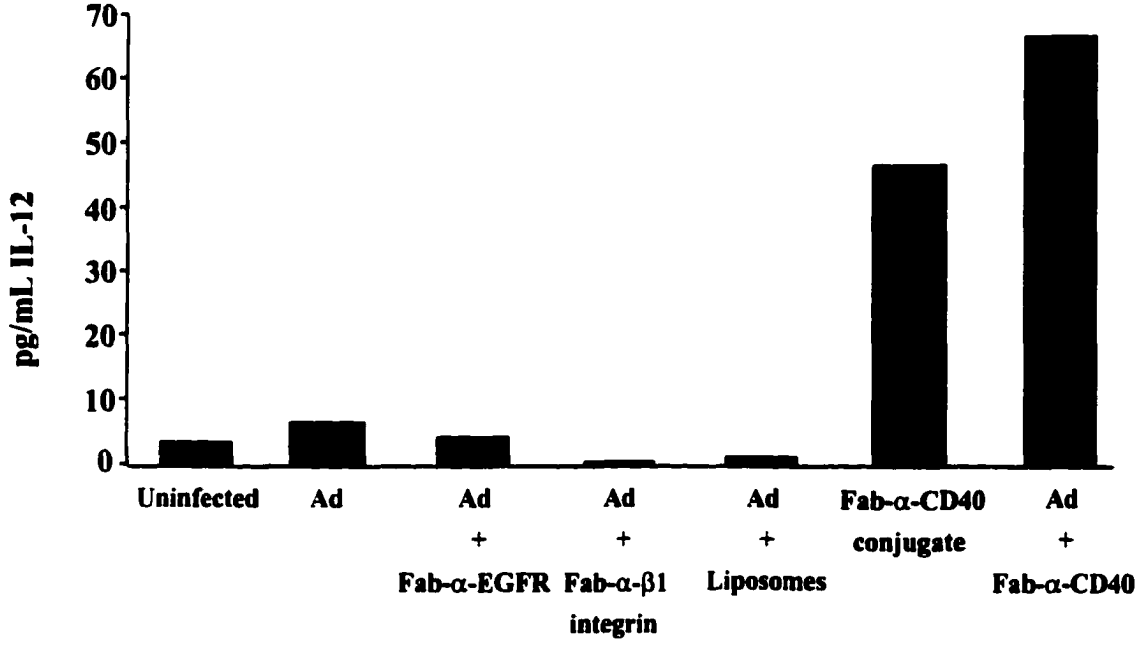

FIGURE 7. IL-12 production is enhanced after treatment with Fab-anti-CD40 conjugate or CD40-targeted Ad. MDDC were treated with the indicated retargeted Ad vectors (MOI 100) or the Fab-anti-CD40 conjugate in the absence of Ad. At 48 h, the supernatants were assessed by ELISA for production of IL-12. Values < 8 pg/ml are beyond the linear range of detection by this assay. A representative experiment of two is shown.

Discussion

Despite enormous clinical potential, widespread application of genetically modified DC has been hindered by several obstacles. Among these are the extensive handling required for ex vivo transduction, the poor gene transfer efficacy by existing vectors, and the necessity to mature DCs to an optimal T cell activation status subsequent to gene transfer (2, 28). With regard to the latter, peripheral DC active in the process of Ag capture are referred to as "immature DC." In spite of active Ag retrieval, these cells do not express the adequate panel of costimulatory molecules and cytokines necessary to activate immune effector cells. Therefore, immature DC must be differentiated to an immunologically potent "mature" state that can optimally stimulate immune cells (3, 25, 26, 29). For this reason, we sought to understand what effects this CD40-targeted Ad vector would have on the maturation status of DC. The ability of the anti-CD40 conjugate to mediate DC maturation in the absence of virus clearly indicates that the maturation phenomenon is Ad-independent. Further, expression of CD83 and ICAM-1, production of IL-12, and improved MLR were observed almost exclusively with treatment of MDDC by Fab-anti-CD40 conjugate or CD40-targeted Ad but not with other Ad vectors tested. Based on this finding, it seems fairly certain that the observed DC maturation is a direct and specific result of CD40 engagement.

In summary, it appears from our results that Ad, as reported for several other viruses (3), mediates minor effects on DC phenotype, but that these effects are seen only when a sufficient number of particles enter each cell, such as by the high-efficiency Ab-

targeted or liposome-complexed Ad based vectors. It is interesting to speculate as to whether the enhanced expression of some costimulatory molecules seen with β_1 integrin-targeted or liposome-complexed Ad is a consequence of the capsid itself entering the cell, of reporter gene expression, or of background Ad gene expression. Since the conjugate, or even the unconjugated anti-CD40 mAb, can mediate agonistic DC maturation in an Ad-independent fashion, it would at first seem needless to introduce an adenovirus into the formula. This would indeed be the case if maturation were the only desired outcome. In instances where delivery of an Ag-encoding gene is desired, however, the only way to attain the requisite level of gene transfer is by appending a targeting conjugate to overcome the deficiency of native Ad-binding receptors on DC. Thus, it seems clear that the conjugate forms a fundamental link between the maturational features of an agonistic anti-CD40-antibody and the gene transfer capacity of an adenoviral vector in a manner that exceeds the value of either component individually.

A CD40-targeted Ad vector exhibits several features that may prove attractive for Ad-based vaccination. By virtue of its highly efficient gene delivery, it should be possible to deliver cryptic Ags that might otherwise not be accessible to the immune system. Also, the restricted gene delivery to cells expressing CD40 may prove advantageous for in vivo approaches when compared with less specific vectors like Ad alone or Ad complexed with liposomes. With regard to the maturational capacity of this vector, it has been reported that activation of DC to maturity renders them resistant to both the effects of DC inhibitory cytokines like IL-10 (2, 30, 31) as well as to direct tumor-induced

apoptosis (32). Further, the capacity with which murine DC can generate an immune response in vivo has been shown to correlate with the degree of their maturation (32). Moreover, based on proposals that CD40 activation may bypass CD4+ T cell help (3, 25, 26), a CD40-targeted Ad might also have applications in cases of CD4+ dysfunction. The dual role of CD40 in this schema as both a surrogate Ad receptor and a powerful trigger of DC maturation may prove useful as a retargeting strategy to this central cell type of the immune system. Regardless, we have recognized the limitations of this Ab-based targeting strategy for intensive clinical applications. For this reason, we are currently pursuing a genetic fusion strategy between the trimeric Ad fiber and the natural ligand of CD40, CD40L, which is also trimeric.

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ADENOVIRAL VECTORS TARGETED TO CD40 ENHANCE THE EFFICACY OF DENDRITIC CELL-BASED VACCINATION AGAINST HPV16-INDUCED TUMOR CELLS

by

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Abstract

Dendritic cells (DC) represent a unique cell stratum from which to initiate antigen (Ag) specific immunity. One of the most challenging obstacles for dendritic cell (DC)-based immunotherapy has been the means by which to convey tumor Ag encoding genes to DC. In this study, we show that adenoviral (Ad) vectors targeted to CD40 by means of bispecific Abs can enhance gene transfer to murine DC. 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 Ag. In particular, the human papillomavirus type 16 (HPV16) E7 Ag represents an attractive target for Ag-specific immunity of cervical cancer. Relative to DC infected by untargeted Ad, DC infected by AdE7 targeted to the receptor CD40 enhanced protection against HPV16-induced tumor cells in a murine model. We have further established that this protection was both Ag specific and CD8+ T cell dependent. Illustrating that Ad-modified DC may employed in repeated vaccination, we report that preimmunization of animals with Ad-infected DC before 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 DC with tumor Ags can elicit productive antitumoral immunity and that enhancements in gene transfer efficacy and/or DC maturation may facilitate this process. Ultimately, the employment of such high-efficiency DC vectors may decrease the input vector dose and, consequently, minimize dose-related vector toxicity.

Introduction

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

In this regard, strategies that employ Ag-pulsed DC have proven remarkably effective at protecting animal models from tumor challenge (2, 4-9). Nevertheless, the most challenging obstacle for DC-based immunotherapy has been the means by which to efficiently convey Ags to DC (10, 11). Adenovirus (Ad) has been previously employed as a vector to murine DC in generation of antitumoral immunity (8, 12-15). The inefficiency of Ad-mediated gene transfer, however, is likely to become problematic for large-scale vaccinations. We have previously shown that retargeting Ad to CD40 on human DC both enhances gene transfer and promotes DC maturation (16). We hypothesized that coupled to Ad vectors carrying genes for tumor Ags, this strategy might enhance the efficacy of DC-based vaccinations. Specifically, we have chosen to explore the utility of this strategy in a murine model of human papillomavirus (HPV)-induced cancer. To this end, E6 and E7 Ags of "high risk" HPV types 16 and 18 are required for maintenance of the malignant phenotype (17, 18), rendering these gene products as unique candidates for Ag-specific immunotherapy. In this study we have chosen E7 because of its previously

reported advantage over E6 for vaccinations (5). A further anticipated obstacle for Ad-modified DC is the prospective efficacy in subjects that have been previously exposed to adenovirus. Therein, we have investigated the potential to vaccinate mice that have been previously exposed to Ad-infected DC.

Here, we describe the use of a CD40-targeted Ad vector carrying the gene of the HPV type 16 E7 Ag for genetic modification of murine DC. Importantly, the E7 gene contains a deletion that renders the oncogenic retinoblastoma binding domain nonfunctional (19). We provide evidence that DC genetically modified by targeted adenovirus can efficiently initiate Ag-specific immunity toward tumors expressing HPV-16 E7. We also demonstrate that targeting of the Ad vector to CD40 imparts an advantage in a vaccination context over untargeted adenoviral vectors. Finally we report that such vaccinations retain their potency despite preimmunization of animals with Ad-infected DC.

Materials and Methods

Mice. C57BL/6 (B6, H-2^b) mice were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were used at 4-8 weeks of age.

Viruses and cell lines. Ad carrying the gene for HPV E7 mutant in the pRb binding domain, indicated in the text as AdE7, was kindly provided by Dr. Pradip Raychaudhuri (University of Illinois at Chicago) (19). Preparation of plaque-titered Ad vectors AdLuc and AdGFP, carrying the gene for luciferase and green fluorescent protein (GFP), respectively, has been previously described (16). The C3 tumor cell line (a kind

gift of Dr. Jan Ter Schegget, University of Amsterdam) was generated by transfecting C57BL/6 mouse embryonic fibroblasts with plasmids containing the entire genome of the HPV16 (20). B16 melanoma cells were obtained from the American Type Culture Collection (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 hybridoma FGK45 (21) was generously provided by Dr. Antonius Rolink (The Basel Institute for Immunology, Switzerland). The neutralizing murine hybridoma 1D6.14 specific for the carboxy-terminal, receptor-binding knob domain of Ad serotype 5 fiber has been previously described (22). These hybridomas were used to generate hybridoma supernatants using Nutridoma (Boehringer Mannheim, Indianapolis, IN). Purification of Abs and Fab fragments has been described previously(16). Bispecific Abs consisting of the 1D6.14 neutralizing anti-Ad knob Fab fragment and the anti-CD40 antibody were prepared by chemical cross-linking with *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) as previously described (23). The conjugate of FGK45 mAb and 1D6.14 Fab is henceforth designated as Fab-anti-murine CD40.

Assessment of phenotypic DC maturation. For maturational analyses, Abs used were directly conjugated to FITC (Pharmingen, San Diego, CA). These included 3E2 (anti-CD54), 16-10A1 (anti-CD80), GL1 (anti-CD86), AF6-88.5 (anti H-2K^b), AF6-120.1 (I-A^b), G155-178 95 (Mouse IgG isotype control), R35-95 (Rat IgG isotype control), and G235-2356 (Hamster IgG isotype control). Anti-CD40 mAb,

FGK45, was detected by the FITC-labeled goat anti-rat mAb (Jackson Immunoresearch Laboratories, West Grove, PA).

Bone marrow-derived DC. Bone marrow DC were prepared as previously described by Inaba (24). Briefly, bone marrow was collected from femurs and tibias of 4- to 8-wk old C57BL/6 mice. Bone marrow cells were incubated with a mixture of Abs 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 containing 10% FCS and 100 U/ml recombinant murine GM-CSF (Peprotech, 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 DC was established by the absence of lineage markers in flow cytometry analysis.

Preparation of targeted Ad. Ad was incubated with an optimal ratio of targeting conjugate as previously described (16). Briefly, Ad was incubated for 30 min at room temperature with Fab-anti-murine CD40 at a ratio of 30 ng:2.4 X 10^6 PFUs in complete RPMI 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 DC for assessment of GFP gene transfer. To assess the percentage of DC transduced, cells plated in 6-well plates were infected with untargeted or CD40-targeted AdGFP at a multiplicity of infection (MOI) of 10, 100, or 1000 in the presence or absence of conjugate for exactly one h at 37°C before unbound virus was washed away with PBS. Cells were subsequently incubated in RPMI 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 DC for maturation analysis and immunizations. Adherent DC were incubated for exactly 1 h at 37°C under one of the following conditions: mock infection (DC), 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.

Prophylactic DC immunization. Mice were administered a primary vaccination intradermally equal to the number of DC indicated; 1 wk 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 among 4-5 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 subcutaneously 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 injected i.p. with 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 day -2, 1, 5, 10, 13, and 17. CD8+ depletion was validated by flow cytometry of splenic suspensions. On day 0, mice received a primary vaccination of 12,000 DC infected as detailed in *Materials and Methods*. Subsequently, on day 7, a booster vaccination of 6,000 DC was administered, and on day 14 a challenge with 2 million C3 was given.

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

Vaccination against established tumors. Tumors were established by subcutaneous injection of C3 cells 3 wk before the first vaccination. Only mice bearing tumors with a minimal volume of 100 mm^3 at 3 wk were advanced to therapeutic vaccination studies. Mice were size matched into four groups corresponding to a group of unvaccinated animals or those vaccinated with DC infected by CD40AdLuc (40AdLuc), AdE7,

or CD40AdE7 (40AdE7). Mice were immunized with a dose of 200,000 DC in a total volume of 200 μl on each of four weekly vaccinations. In particular, mice were vaccinated at four to five sites distant from the tumor mass. Tumors were monitored for 15 weeks or until tumors had reached a volume of 1000 mm^3, at which point mice were euthanized.

Statistical analysis. The chi-square 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 DC. A limited availability of efficient strategies to deliver Ag encoding genes to DC has hindered gene-based DC vaccination strategies. We have previously illustrated high-efficiency gene transfer to human DC through targeting of Ad to CD40 by means of bispecific Abs. Subsequently, we have adapted 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 DC relative to untargeted Ad, delivery of the marker gene GFP by Ad was assessed by flow cytometry. As shown in Fig. 1*A*, CD40-targeted Ad demonstrated enhanced gene transfer relative to untargeted Ad at each MOI tested. At an MOI of 100,

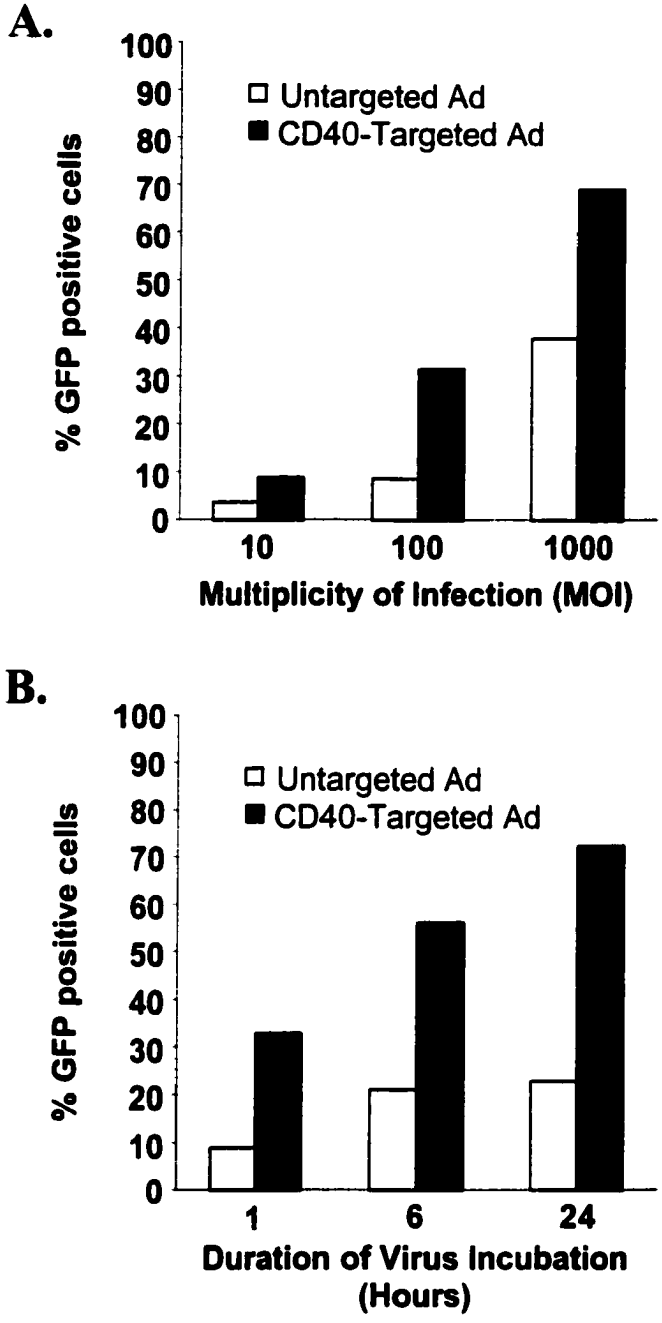

FIGURE 1. Targeting of Ad to CD40 enhances the number of cells transduced relative to untargeted Ad. Murine bone marrow-derived DC were infected with AdGFP either alone or complexed with Fab-anti-CD40 (*A*) for exactly 1 h at an MOI of 10, 100, or 1000 (*B*) for 1, 6 or 24 h at a constant MOI of 100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

for instance, CD40-targeted Ad transduced 30% of cells, compared with only 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. In contrast to our finding of poor gene transfer with Ad in the absence of targeting, others have reported a high efficiency of gene transfer to DC by at similar dosage (15, 25). To reconcile our findings with these reports, we examined the possibility that more cells may be transduced following extended duration of viral incubation. As shown in Fig. 1*B*, extended exposure of cells to virus yielded a higher percentage of DC 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 tested. These higher levels of gene expression following 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 DC relative to untargeted vector.

CD40-targeted Ad phenotypically matures murine DC. We have previously described phenotypic maturation that accompanies infection of human DC by CD40-targeted Ad (16). The essential role of maturity in the activation of T cells (1, 2) suggests that DC 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, DC that had been infected with untargeted Ad or CD40-targeted Ad were compared to uninfected cells by flow cytometry (Fig. 2). Compared to uninfected cells, cells infected by CD40-targeted Ad enhanced expression of several

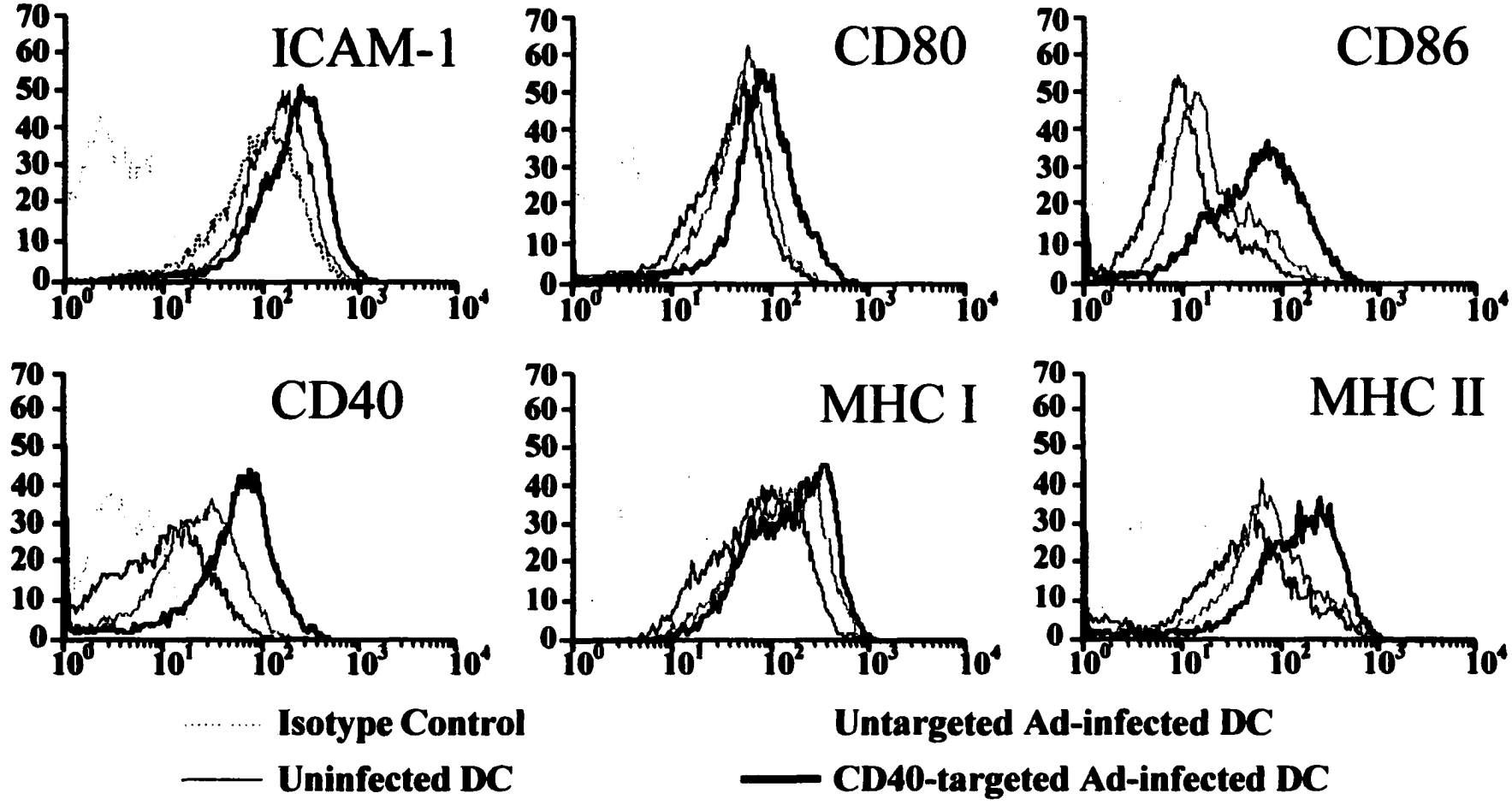

FIGURE 2. CD40 targeting induces expression of DC maturational markers. DC were infected by mock infection, untargeted AdLuc, or CD40-targeted AdLuc for 1 h at an MOI of 100 and subsequently incubated for 24 h before to analysis. Samples shown in-dicate expression of CD54, CD80, CD86, CD40, MHC I, and 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.

markers associated with DC maturation, particularly CD40, CD86 and MHC II. Minor changes were observed for cells infected with untargeted Ad, but these were of smaller magnitude 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.

DC modified by CD40-targeted Ad exhibit enhanced vaccination potential. To establish the efficacy of Ad modified DC for immunization, we have employed the syngeneic C3 tumor model of HPV-induced neoplasms (26) and a functionally mutated gene for the E7 Ag of HPV within an Ad vector, AdE7 (19). 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. DC infected ex vivo with an MOI of 100 were administered intradermally in primary and secondary vaccinations set a week apart. Specifically, DC were titered such that mice received a primary vaccination of 25,000; 12,500; or 6250 DC as indicated in Fig. 3, followed by a booster vaccination 1 wk later using half the number of cells employed in the primary vaccination. A week after the booster vaccination, mice were challenged s.c. with 2 million C3 tumor cells. These findings reveal that by a dose of 12,000 DC, for example, tumors had developed in animals vaccinated with DC transduced by untargeted AdE7 but not when CD40AdE7 had been employed (Fig. 3). Of note, among the tumors that did develop on mice in the lower dosage classes of E7- modified DC, the kinetics of tumor growth were slower than in mice that had been left unvaccinated (data not shown). These findings suggest that DC modified to express tumor Ag by Ad vectors can mediate

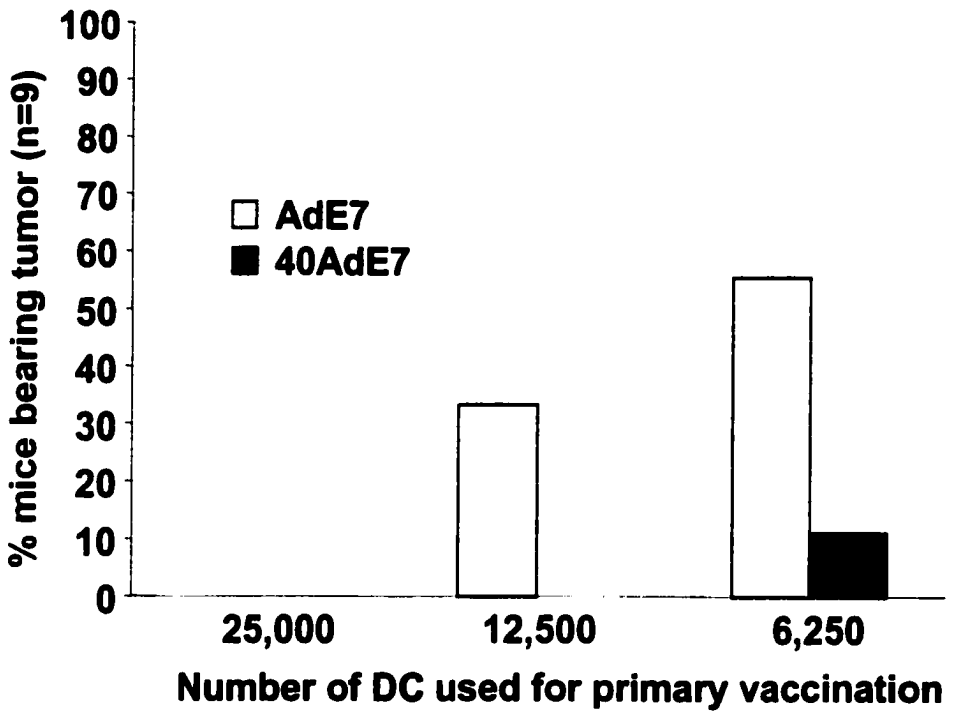

FIGURE 3. DC infected by CD40-targeted Ad exhibit an advantage for in vivo vaccination over DC infected with untargeted Ad. Mice were vaccinated by intradermal injection of graded doses of DC infected by either untargeted (AdE7) or CD40-targeted AdE7 (40AdE7) as shown. On day –14, animals received a primary vaccination of 25,000; 12,500; or 6,250 DC 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 post-tumor challenge is shown in this representative experiment. Analysis demonstrated significantly enhanced protection in mice vaccinated with 40AdE7 relative to mice vaccinated with AdE7. ($p < 0.05$)

dose-dependent prophylactic protection to tumor challenge and, more importantly, that features of CD40-targeted Ad translate to an advantage for vaccination.

E7-based vaccination is Ag specific. DC impact the immune system through a number of Ag-nonspecific mechanisms (1). To establish that tumor protection was specific for E7 Ag, two avenues were investigated. First, a control vector (AdLuc), carrying the gene for an irrelevant Ag, luciferase was employed. Alternatively, a tumor line, B16 melanoma cells, negative for expression of the E7 Ag, was used in place of C3 cells for tumor challenge. As controls for nonspecific immune activation, DC were left uninfected or infected with CD40-targeted irrelevant vector AdLuc. Mice were vaccinated with DC infected with the indicated vector by a primary vaccination of 12,500 DC, followed by a booster vaccination of 6,250 DC seven days later. A week after the booster vaccination, mice were challenged with 2 million C3 tumor cells or 20,000 B16 cells, as shown. While unvaccinated mice developed C3 tumor masses, mice vaccinated with AdE7 transduced DC did not develop tumors (Fig. 4). Importantly, the baseline percentage of mice developing C3 tumors in unvaccinated mice is less than 100%, as reported previously (5). Notably, both unmodified DC and AdLuc-transduced DC imparted minor but not significant protection against tumor development. Alternatively, DC transduced with AdE7, whether targeted or not, were unable to protect mice from challenge with Ag disparate B16 melanoma. These findings illustrate that DC genetically modified by targeted Ad generate immunity that is Ag specific as defined by the transgene carried within the adenoviral vector.

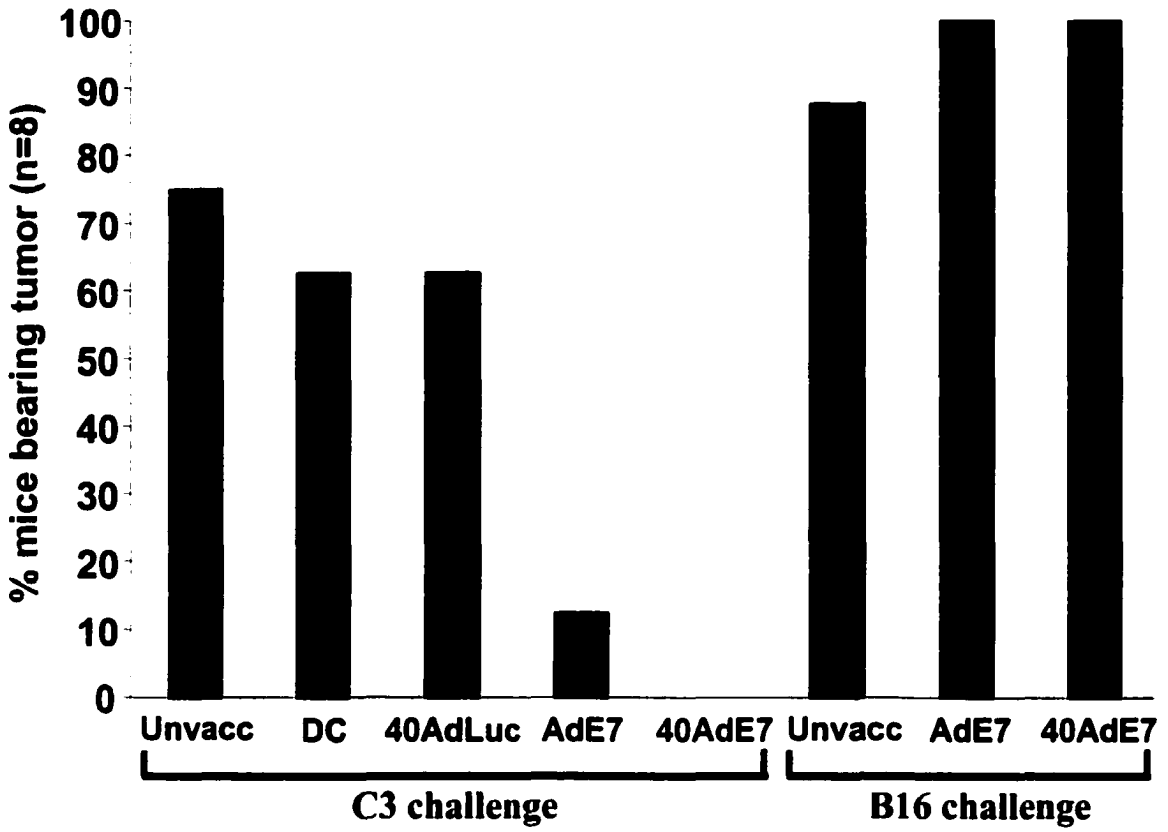

FIGURE 4. DC genetically modified by Ad vectors elicit Ag-specific immunity. Animals were left unvaccinated (Unvacc) or vaccinated by intradermal injection with DC infected as follows: mock infected (DC), CD40-targeted AdLuciferase (40AdLuc), untargeted AdE7 (AdE7), or CD40-targeted AdE7 (40AdE7) as described in *Materials and Methods*. Except for unvaccinated animals, each mouse received a primary vaccination of 12,500 DC and a booster vaccination of 6,250 DC at 14 and 7 days before 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 indicating the percentage of mice bearing tumors at 6 wk is shown.

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 DC are believed to mediate their effects on antitumor immunity (27). To investigate the role of CD8+ T cells in the tumor protection observed with this system, subsets of mice were depleted of CD8+ T-cells during primary and booster vaccinations and subsequent tumor challenge with C3 tumor cells. While 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 DC infected either by untargeted or CD40-targeted Ad is mediated through CD8+ T cells.

Preimmunization with Ad-infected DC 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 (28-30). To examine the potential that Ad-transduced DC may compromise subsequent administrations, mice were preimmunized by primary and booster vaccinations of DC infected by Ad carrying a gene for an irrelevant Ag, luciferase. Subsequently, mice were administered primary and booster vaccinations of AdE7-transduced DC at 1 and 2 wk after preimmunization, respectively. To enhance the stringency of this pre-immunization, the doses of DC in primary and booster vaccinations for AdLuc-infected DC were twice the doses of subsequent E7-modified DC. One week after the final immunization, mice received a tumor challenge with C3 cells. In mice vaccinated with 40AdE7-infected DC, preimmunization with AdLuc-infected DC resulted in tumor growth in 30% of animals, compared with complete protection in mice that had not been preimmunized (Fig. 6).

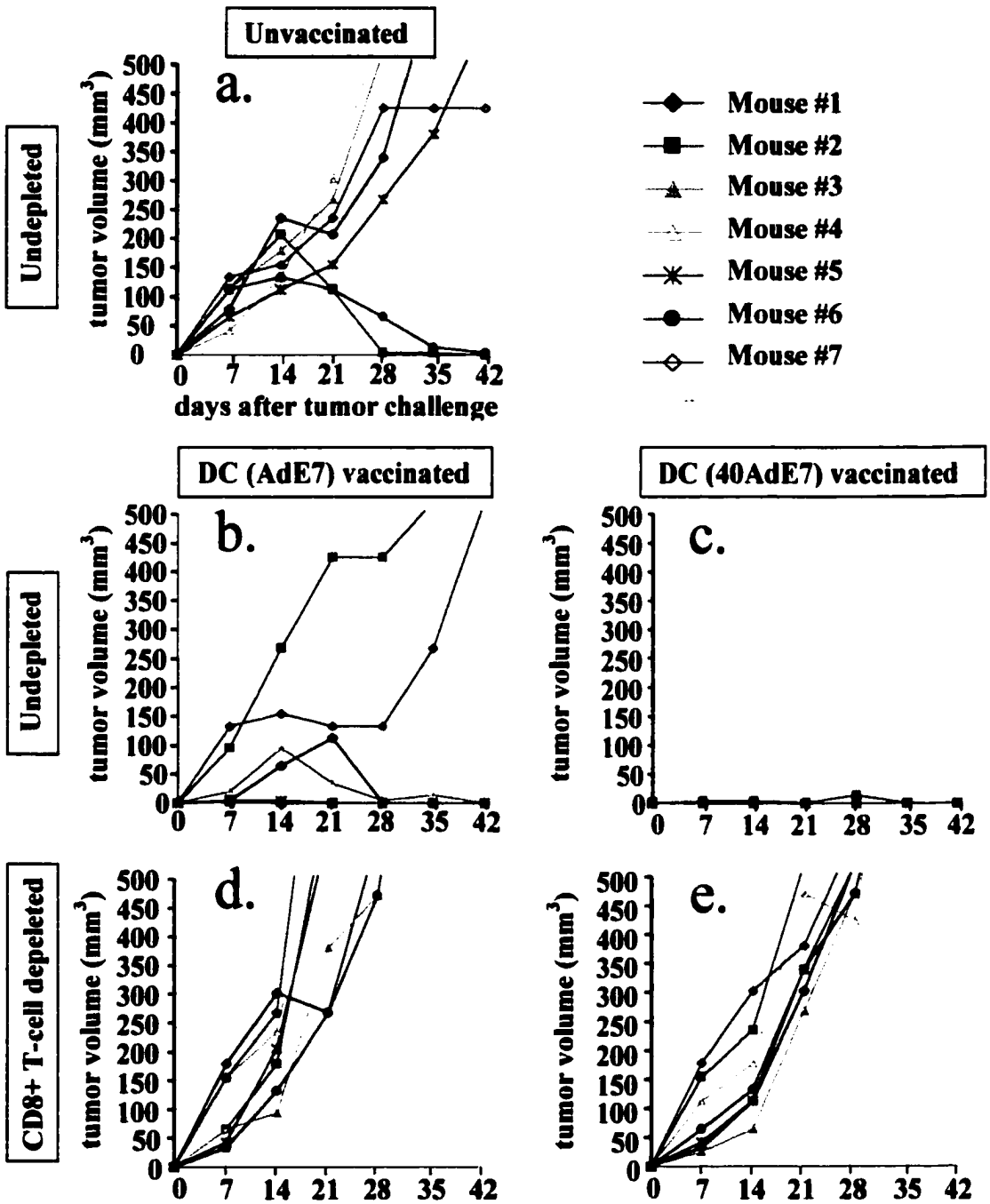

FIGURE 5. Immunization with Ad-modified DC is CD8+ T cell dependent. CD8+ T cells were depleted in vivo with mAb (*d* and *e*) as described in *Materials and Methods*. Mice were left unvaccinated (*a*), or immunized with DC previously infected by untargeted AdE7 (*b* and *d*) or CD40AdE7 (*c* and *e*) in primary and booster doses of 12,500 and 6,250 DC, respectively. Tumor growth per each condition is shown for 6 wk after tumor challenge or until the tumor volume exceeded 500 mm^3.

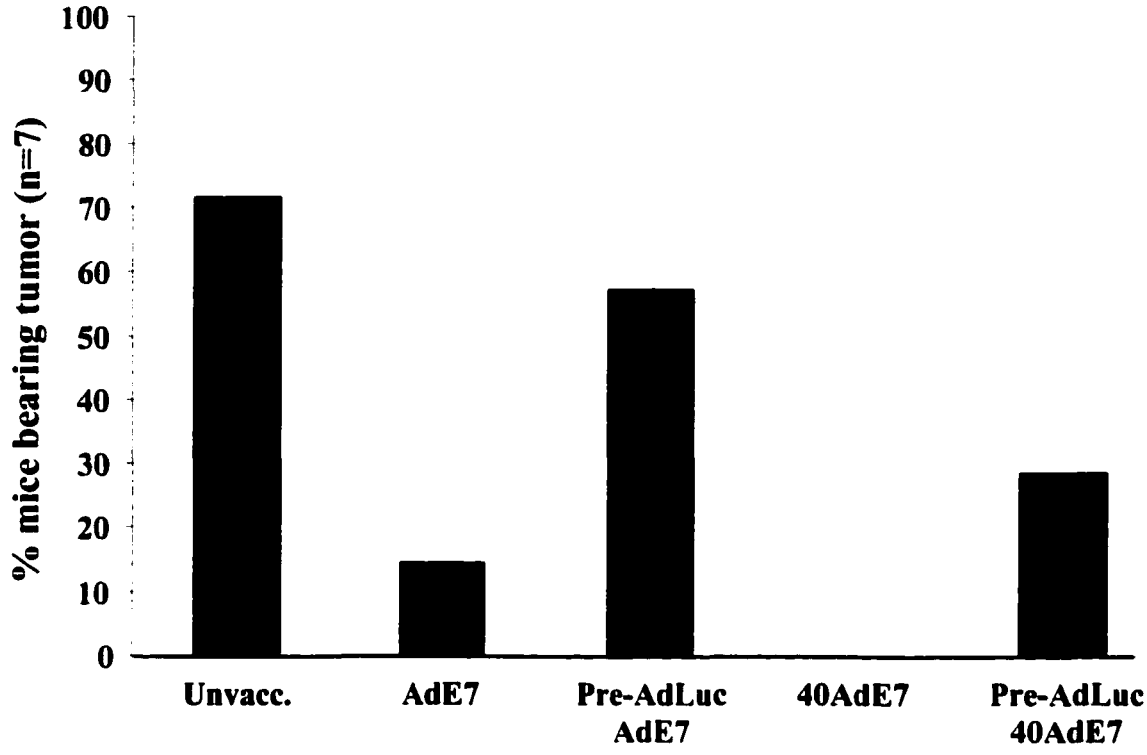

FIGURE 6. Preimmunization with Ad-infected DC partially reduces the efficacy of Ad-modified DC vaccines. Mice designated with the prefix "Pre-AdLuc" received a primary prevaccination of 25,000 AdLuc-infected DC and a booster of 12,500 AdLuc-infected DC 28 and 21 days before tumor challenge, respectively. At 14 and 7 days before tumor challenge, mice received vaccinations of 12,500 and 6,250 DC, respectively, infected with either untargeted Ad (AdE7) or CD40-targeted Ad (40AdE7), as indicated. Mice were challenged s.c. with 2 million C3 cells. The percentage of mice bearing tumors is shown at 6 wk after tumor challenge.

These findings suggest that DC may be administered on multiple occasions and yet still provide protection in a significant percentage of preimmunized animals.

DC 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 DC 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. Tumor-bearing mice remained unvaccinated or were administered four equivalent doses of 200,000 Ad-modified DC spaced at weekly intervals with DC that had been infected by CD40-targeted AdLuc, untargeted AdE7 or CD40-targeted AdE7, as indicated. As shown in Fig. 7, compared to unvaccinated animals, mice vaccinated with DC infected by CD40-targeted AdE7 were able to significantly delay continued growth and ultimately extended survival. In contrast, tumor growth in mice vaccinated with AdLuc-transduced DC was not significantly distinct from growth in unvaccinated animals. These findings confirm that genetically modified DC can initiate an Ag-specific therapeutic immune response against E7.

Discussion

The potential utility of genetically modified DC is evidenced by their proposed applications in the treatment of infectious diseases, autoimmunity, allotransplantation, and cancer (1, 31). A significant hurdle to large-scale application of therapies using DC will be a means by which to efficiently deliver Ag-encoding genes to these cells. In this

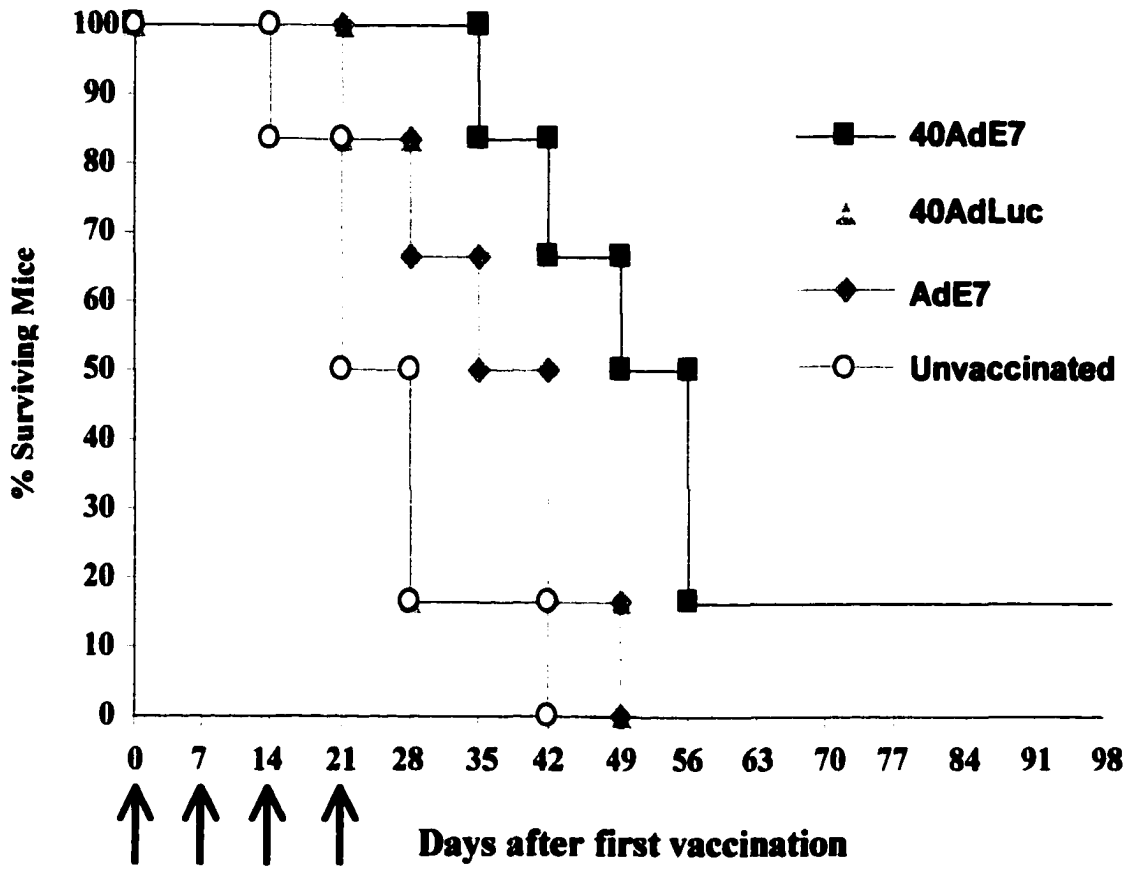

FIGURE 7. DC infected with AdE7 can mediate therapeutic tumor immunity to extend survival of animals with pre-established tumors. Groups of animals bearing size-matched, established C3 tumors were left unvaccinated or were immmunized with DC infected by one of the following vectors as indicated: CD40-targeted Ad Luciferase (40AdLuc), untargeted AdE7 (AdE7), or CD40-targeted AdE7 (40AdE7). Four weekly vaccinations of 200,000 DC (indicated by arrows) were administered intradermally at sites distant from the tumor mass. The percentage of surviving mice is shown until 14 weeks. Mice were considered deceased when tumor volumes exceeded 1000 mm^3 and were euthanized to avoid unnecessary suffering. Statistical analysis by the log-rank test revealed statistically significant enhancement in survival time for mice vaccinated with 40AdE7-infected DC compared to unvaccinated mice. ($p < 0.05$)

regard, to explain poor infection of human DC, we have previously reported a deficiency of the Ad-binding receptor (16). To exploit the expanding role of CD40 in DC function (32-35) as an alternative Ad-binding strategy, we have coupled a CD40-activating antibody 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 DC and explore the utility of this approach in Ag-specific vaccination.

We have observed that Ad targeted to CD40 consistently demonstrated a greater magnitude of gene transfer than did untargeted Ad. By comparison, our results reveal that untargeted Ad tranduces a mere 8% of murine DC at an MOI of 100; these findings are consistent with those of several reports (12, 36). In contrast, some investigators describe transduction efficiencies upwards of 90% using a similar dose of untargeted virus (15, 25). To this end, it has been previously illustrated that upwards of 80% of virions can localize to a cell's nucleus within 60 min of infection (37); thus, it would seem that gene transfer that occurs on a longer timescale 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 DC 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 hours 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.

The duration of incubation is perhaps inconsequential for ex vivo modification of dendritic cells. Nevertheless, the practical advantages of ultimate in vivo DC transduction are promising, especially in light of recent data suggesting that Ad targeted to CD40 can selectively transduce Langerhans cells of human skin (S. Luykx-deBakker, manuscript in preparation). Accordingly, in vivo vaccination would eliminate the necessity for ex vivo manipulations to DC, 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 important, 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 (38-42).

We also provide evidence of phenotypic maturation in murine DC 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 (33, 35, 43). 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 (43, 44). 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 Ag presentation through DC maturation.

To establish whether DC modified ex vivo imparted an advantage in vivo, we have compared the vaccination potential of DC 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 than untargeted Ad and in an Ag-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 has prompted the employment of peptide-loading approaches for DC-based vaccinations (2, 31), which have included approaches directed toward HPV-E7 (43, 45-47). 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 heterogenous MHC alleles and further by the narrow range of epitopes provided by individual peptides (48). Such limitations are likely to be obviated through the use of gene-based modification of DC. By delivery of the E7 gene in its entirety, DC can present from among a vast array of potential epitopes that are appropriate for the MHC alleles of the recipient. The basis of E7 oncogenicity has been defined (49, 50), and thus we have employed a mutant rendered functionally inoperative in its oncogenic pRb binding domain (19). It is also important to recognize that E7 expression alone is not sufficient for malignancy (51) and, further, that transformation is dependent upon continuous expression of E7 (52). 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 oncogenes need not necessarily be viewed with skepticism.

Legitimate concerns have been raised about the utility of Ad vectors in a population that has been previously exposed to adenovirus (53, 54). Indeed, anti-Ad cellular immune responses have been recognized to severely compromise the duration of gene expression (28-30, 55-57). In particular, Jooss et al. have shown that anti-Ad immune responses are a consequence of Ad transduction of DC (58). In this regard, it would seem that DC intentionally modified by adenoviral 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 DC for vaccination despite prior immunization with infectious Ad particles (12, 13). We reasoned that rather than isolated Ad particles, preimmunization with Ad-infected DC would more rigorously test the capacity of Ad-infected DC for repeated administration. Our findings reveal that pre-immunization with Ad-infected DC 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 (29). By comparison, the time frame for both migration of DC to lymphoid organs and interaction of DC with T cells occurs much more rapidly (59, 60). Further to this end, it has been established that DC undergo apoptosis after interaction with T cells (61); thus, it would seem that long-term expression of Ags is not requisite for initiation of a productive immune response. We hypothesized that activation of T cells by Ad-infected DC may fall within a window before immune clearance of infected cells. While our studies cannot conclude that repeated administrations will remain efficacious indefinitely, they do suggest that DC 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 DC 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 Ag presentation can effectively generate immune responses, which would then be rendered enduring not by the DC, 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 rigorously demonstrated. We have vaccinated mice bearing palpable pre-established tumors with DC infected by Ad carrying the gene for E7 or an irrelevant Ag. Our findings indicate that despite a significant prolongation in survival in animals vaccinated with DC 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 suggests that 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 Ag; alternatively, these cells may have undergone an "immunological escape" *in vivo*, much as human tumor cells tend to do (27). These findings suggest that an optimal vaccine will poten-

tially incorporate several Ag genes within a single vector, thus minimizing the potential for such escape.

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 Ag-specific manner. Further, we conclude that Ad-transduced DC may be administered in a limited number of repeated doses without entirely compromising vaccine efficacy.

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SUMMARY

As cancer has remained refractory to pharmacologic and conventional vaccines alike, the endeavor of novel treatment approaches has become appropriate. The field of gene therapy in particular has presented regulated and relatively tumor-specific alternatives. Whereas, collectively, the definition of specific molecular defects has expanded the understanding of cellular transformation, individually, each of these defects may compromise but a small percentage of total cancers (17). Thus, individual agents targeted to these defects may act in too narrow a spectrum to be implemented on a large scale. Even when more prevalent defects are identified, direct gene transfer to cancer cells will most certainly never permeate the host adequately to reach every tumor cell. Therefore, in gene therapy of individual cancer cells risks incomplete tumor cell eradication and the potential for relapse. In contrast, the adept nature of the immune system at generating systemic protection more completely ensures that tumor cells will be eradicated. In particular, the concept of a vaccination against a panel of the most common tumor Ags may be envisioned as a means to treat cancers with different underlying causes.

The failure of conventional vaccines to provide protection against cancer has been a powerful incentive to endeavor fundamentally new modes of vaccination. In this regard, the advent of technologies to manipulate DC has shuttled DC-based therapy to the forefront of novel therapeutic strategies. In particular, the evidence suggests that the route by which Ags are delivered ultimately affects the type of immune response

generated. Soluble vaccines, for instance, are appropriate for generating humoral response; yet, while antibodies are aptly suited for the control of extracellular pathogens, this arm of immunity is markedly less effective against virally infected or transformed cells. More importantly, the preferential display of extracellular soluble Ags in the context of class II MHC precludes the efficient activation of class I-restricted CD8+ cytotoxic T-lymphocytes (176). As the limiting agent in antitumoral immunity, the absence of sufficient CTLs may explain the poor response rates observed with cancer vaccines in the conventional format. Alternative means to route Ags specifically to class I compartments have been established. Particulate Ags, for instance, increase the extent of "cross-priming" of extracellular Ags onto class I molecules (21). Likewise, viruses and intracellular bacteria can express genes within the cell, and these gene products are processed for Ag presentation, presumably through the conventional class I pathway (17). In essence, any of these agents could serve as a vehicle for Ag encoding genes. Nonviral DNA delivery to DC by liposomes (179) or particle bombardment (180) has suffered from poor efficiency of gene transfer. The concept of a virus that delivers genes to DC, which are subsequently expressed and presented as Ags, is not without precedent. It is known that DC infected with influenza virus sustain continued expression of the viral gene products rather than commit suicide as the fate of other cell types would tend to suggest (181). In this manner, DC act as an altruistic host to present gene products of the influenza virus to immune effector cells, thus facilitating immune clearance of the virus from the host. Viruses, in particular, have been used extensively for the purpose of gene transfer and represent the most readily available option.

Viruses by their very nature are evolutionarily designed with many features conducive to the delivery of genes. Viral structural proteins, for example, are instrumental in directing the virus to an appropriate host cell. Moreover, once inside a cell, carefully timed dismantling of the capsid protects the viral genome against the harsh endocytic environment and yet disassembles at the proper moment to permit gene delivery. So effective are these agents at efficient gene transfer that many nonviral vectors have been modeled after their viral counterparts. By far the primary drawback of viral and nonviral vectors alike has been their lack of cell-specific targeting. The advent of new technologies to specifically target these vectors promises to greatly increase cell-specific gene transfer.

In this regard, the use of bispecific conjugates to target Ad vectors has demonstrated the capacity to mediate gene delivery with greatly improved specificity even in the absence of native Ad-binding receptor (132, 143, 182, 183). Relevant to gene delivery in dendritic cells, our findings have shown that a similar strategy can enhance gene transfer to DC, presumably by bypassing an established deficiency of the primary Ad binding receptor (Fig. 4). Likewise, the maturation status of DC is central to their ability to present Ags. Our findings revealed that targeting Ad to CD40 was accompanied by improved cytokine production as well as enhanced T cell stimulatory capacity. In recognizing that both T cells and DC are susceptible to tumor-induced dysfunction, it has also been shown that reciprocal exchange of signals between the two cell types may promote survival of each (18). CD40 in particular has been highlighted as one of the more prominent of these signals. For both T cells and DC, interaction of CD40-ligand with CD40 imparts resistance to Fas ligand-induced apoptosis. Indeed, a number of

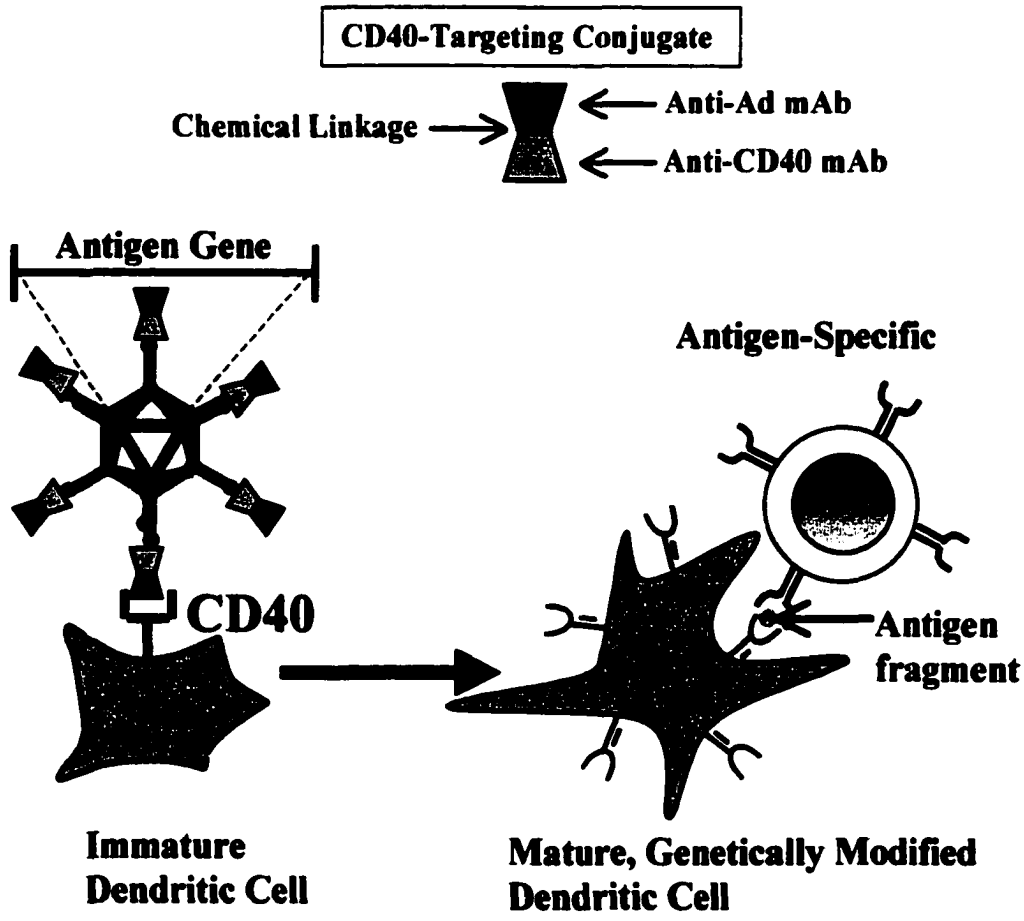

FIGURE 4. Genetic modification of DC by targeted adenoviral vectors to initiate T cell immunity. Targeting of Ad to CD40 enables high-efficiency modification of DC; simultaneous CD40-induced maturation enables efficient Ag presentation to Ag-specific CTL.

studies have highlighted significant improvements in vaccine approaches when used in conjunction with CD40 activation (184). Thus, the role of maturation serves a dual purpose in DC-based vaccination, through both enhanced Ag presentation and resistance to apoptosis. Unlike natural pathogen-infected cells, cancer cells do not generate immune potentiating danger signals, and immune effector cells are not effectively activated (17). In this regard, CD40 activation may supplant the otherwise absent danger signals to promote immune activation. The concept of CD40-induced maturation is by no means novel, but coupling this feature in a manner that simultaneously enhances otherwise poor gene transfer of Ad vectors surmounts one of the more problematic goals in DC-based therapy, namely intracellular Ag delivery.

Importantly, our experiments have not explicitly resolved whether the enhanced gene transfer or maturation is more important from an ex vivo vaccination perspective. Indeed, numerous factors could affect vaccine efficacy based on maturational state ranging from migrational differences to extended lifespan. In any case, from a gene transfer perspective, any feature that reduces the input viral dose should ultimately reduce the complications associated with viral toxicity. CD40 targeting, for instance, can reduce the dose needed to attain a given percentage of transduced cells by at least 5-fold, in the human and murine systems. Such enhancement could have enormous implications for reducing viral toxicity.

The success of this approach suggests this system might bypass key functional obstacles to traditional soluble-based vaccinations. Most likely, the earliest applications of DC-based therapy will be therapeutic in nature; yet, our findings indicate that despite initial prologations in survival of tumor-bearing animals, a vast majority eventually

succumb to the tumor. The failure of E7-based vaccination to mediate complete tumor regression might be explained in several ways. The extended survival suggests that an immune response is initiated, but subsequently compromised or rendered ineffective. In particular, the tumor cells used in these experiments were not maintained under a selective pressure. It is not unreasonable that subpopulations of these cells did not express the E7 tumor Ag; alternatively, these cells may have undergone an "immunological escape" in vivo, much as human tumors tend to do (17). These findings suggest that an optimal vaccine will potentially incorporate several Ag genes within a single vector, thus minimizing the potential for such escape.

Antivector immunity has been an arena of much criticism for Ad vectors. The concept that Ad-infected cells will be eradicated has greatly discouraged the use of Ad vectors for long-term genetic correction. This pitfall is only worsened by the inherently transient gene expression by the nonintegrative Ad vector. The failure of Ad in long-term gene therapy should not exclude its consideration in other applications, however. In particular, the relatively rapid translation of Ag presentation into the activation of T cells suggests Ad-modified DC might act before immune clearance of these cells (147, 148). Accordingly, the findings described herein propose that even mice preimmunized with Ad-infected DC are still susceptible to vaccination by Ad-modified DC. Thus, DC may represent an attractive niche for an otherwise efficient gene vector.

The primary advantage of the Ab targeted system proposed herein is flexibility. Specifically, the Ab based approach can be employed with any Ad vector with Ad type 5 capsid. Thus, this approach might be used to rapidly screen tumor Ags for antitumoral immunization efficacy. In the long term, however, bispecific antibodies exhibit several

disadvantages from a clinical standpoint. First, the intrisic heterogeneity of the chemical conjugates used in these studies may prove problematic for clinical applications. One means to obviate the chemical conjugation involves a genetic fusion between proteins with affinities for the Ad fiber and CD40, either in the form of recombinant Abs or their native ligands (Fig. 5); this would enable production of homogenous Ad targeting entities in a large-scale bioreactor system. Second, the inherently noncovalent association of the conjugate with the viral capsid means that the conjugate may become detached from the virion. This is not only counterproductive to efficient gene transfer to the cell type of interest, but also increases the potential for ectopic gene transfer to cells within the native Ad tropism. Ultimately, the efficacy and specificity of this approach will be greatly improved if targeting ligands can become genetically incorporated into the Ad capsid to create a single-unit targeted vector. Work currently underway within the Curiel laboratory has demonstrated the capacity to append targeting ligands while retaining essential conformational restrictions of the Ad fiber. These advancements will surely expedite development of such genetically targeted viral vectors.

The inherent differences between the murine and human immune systems are indisputable. Even on a microscale level we have observed differences between human and murine DC with regard to both the level of maturation of cultured DC and the baseline susceptibility to gene transfer by untargeted Ad. Thus, the findings from the murine trial may fare better or worse in a human system. Furthermore, fundamental differences exist between naturally occurring cervical cancer and the murine tumor line employed here. Histologically, the murine line is composed of mouse embryonic cells (185), while

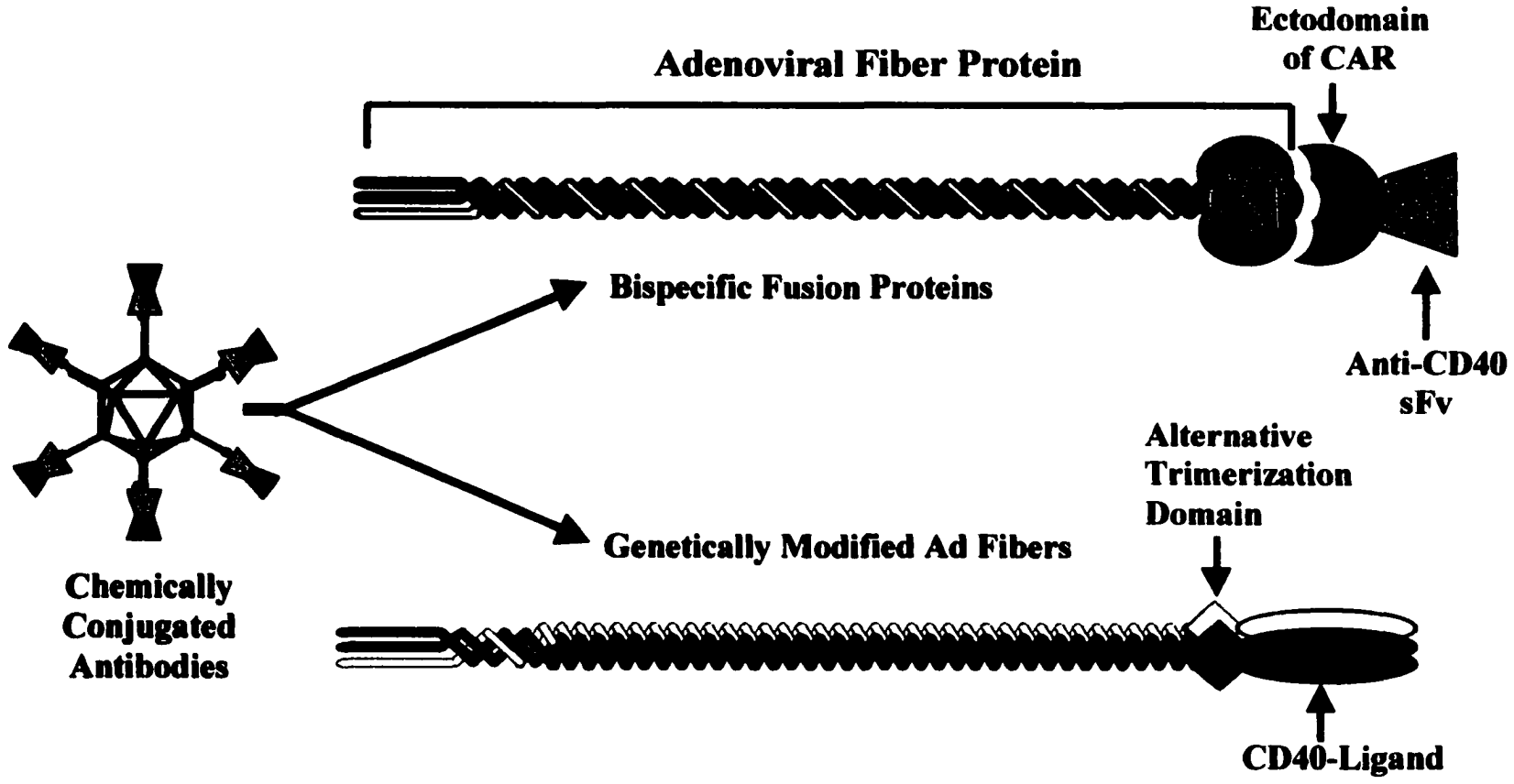

FIGURE 5. Future directions for CD40 targeting of adenovirus. Several limitations compromise the potential for chemically conjugated Abs for large-scale vaccinations. As an alternative, bispecific fusion proteins consisting of an adeno-viral knob binding domain [such as the native Ad receptor CAR or an anti-fiber single chain antibody (sFv)] could be linked genetically to a CD40-binding motif. Optimally, an adenoviral vector with a genetically incorporated targeting domain to CD40 would enable production of single-unit targeted vectors.

cervical cancer is epithelial in nature (153). On the optimistic side, HPV was used to transform the murine line, and a "human" HPV E7 gene was used to vaccinate mice; thus, the transition to a human system may encounter "less refitting" of the components. Moreover, the demonstration that the CD40-targeting approach is functional from its most basic aspects in vitro for human cells provides some level of reassurance from a vector perspective. Despite these findings, many boundaries of genetically modified DC, in particular Ad-modified DC, have yet to be defined. Issues such as the optimal route of delivery, and the dose and frequency of boosters, among others, will markedly influence the success of these vaccinations.

A particularly attractive alternative to the ex vivo-modified DC described here is direct Ad vector administration in vivo. In particular, the demonstration of relative DC specific gene transfer following in situ vaccination by direct intradermal injection as illustrated at the Vrije University of Amsterdam (Luykx-deBakker, manuscript in preparation) may someday eliminate the need for ex vivo modification of DC. This application would greatly enhance the simplicity and practicality of the system, particularly on a global scale, where facilities for culture of DC may be either inadequate or nonexistent.

In the arena of DC-based therapies, the Ad serves merely as a tool by which to generate genetically modified DC. The role of the Ad itself in the future of DC-based therapies is uncertain; most certainly the enduring feature of this work is to illustrate that gene-based immunotherapy, specifically for HPV-induced tumors, is feasible. From a larger perspective, new developments in vaccines may have implications far beyond the treatment of cancer. The current understanding of DC in curtailing untoward immune reponses heralds their potential clinical utility in the prevention of allergy, autoimmunity

and allo- or xeno-transplantation rejection (22). To this end, the public health benefits to be realized are, in and of themselves, almost inconceivable in magnitude. Whether by the methods described here or otherwise, one point is clear: the implementation of genetically modified DC will dramatically impact the field of medicine and public health.

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APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMMITEE APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Office of the Executive Vice President and Provost
Institutional Animal Care and Use Committee

MEMORANDUM

DATE: January 27, 1999

TO: David T. Curiel, MD
Medicine
WTI-614 zip 3300
FAX 975-7476

FROM: Clinton J. Grubbs, PhD
Chairman, Institutional Animal Care and Use Committee

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 1/26/99.
Title of Application: **Vaccination Against Human Papillomavirus Induced Tumors by Use of a CD40 Targeted Adenovirus Encoding Tumor Antigen**
Fund Source: **Internal**

This institution has an Animal Welfare Assurance on file with the Office for Protection from Research Risks (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

CJG/mlw

GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM
DOCTOR OF PHILOSOPHY

Name of Candidate Bryan Walter Tillman

Major Subject Pathology

Title of Dissertation CD40-Targeted Adenoviral Vectors To Potentiate Dendritic Cells For Antigen-Specific Vaccination

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that _he may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

Name		Signature
David T. Curiel, M.D.	**Chair**	
Peter D. Burrows, Ph.D.		
Candece L. Gladson, M.D.		
Jay M. McDonald, M.D.		
Mark J. Mulligan, M.D.		

Director of Graduate Program

Dean, UAB Graduate School

Date 3/23/00