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REVIEW

Combinational adenovirus-mediated gene therapy and dendritic cell vaccine in combating well-established tumors

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Recent developments in tumor immunology and biotechnology have made cancer gene therapy and immunotherapy feasible. The current efforts for cancer gene therapy mainly focus on using immunogenes, chemogenes and tumor suppressor genes. Central to all these therapies is the development of efficient vectors for gene therapy. By far, adenovirus (AdV)-mediated gene therapy is one of the most promising approaches, as has confirmed by studies relating to animal tumor models and clinical trials. Dendritic cells (DCs) are highly efficient, specialized antigen-presenting cells, and DC-based tumor vaccines are regarded as having much potential in cancer immunotherapy. Vaccination with DCs pulsed with tumor peptides, lysates, or RNA, or loaded with apoptotic/necrotic tumor cells, or engineered to express certain cytokines or chemokines could induce significant antitumor cytotoxic T lymphocyte (CTL) responses and antitumor immunity. Although both AdV-mediated gene therapy and DC vaccine can both stimulate antitumor immune responses, their therapeutic efficiency has been limited to generation of prophylactic antitumor immunity against re-challenge with the parental tumor cells or to growth inhibition of small tumors. However, this approach has been unsuccessful in combating well-established tumors in animal models. Therefore, a major strategic goal of current cancer immunotherapy has become the development of novel therapeutic strategies that can combat well-established tumors, thus resembling real clinical practice since a good proportion of cancer patients generally present with significant disease. In this paper, we review the recent progress in AdV-mediated cancer gene therapy and DC-based cancer vaccines, and discuss combined immunotherapy including gene therapy and DC vaccines. We underscore the fact that combined therapy may have some advantages in combating well-established tumors vis-a-vis either modality administered as a monotherapy.

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Introduction

A number of different approaches have been developed to generate antitumor effects against neoplasms. Extensive studies have shown that cancer is caused by genetic mutations that are responsible for the neoplastic phenotype of malignant cells [1]. Therefore, it stands to reason that cancer should also be amenable to correction through gene transfer approaches. It thus comes as no surprise that, to date, gene-based cancer treatment protocols have dominated gene therapy trials. In fact, according to the 2004 report of the National Institutes of Health Recombinant DNA Advisory Committee, cancer gene therapy accounts for more than two-thirds of all the gene therapy protocols that have been reviewed [2].

The critical step for the success of gene therapy is the development of an efficient vector system that is able to specifically deliver therapeutic genes into the targeted cells. Up to now, adenovirus (AdV)-mediated gene therapy has been shown to be an attractive approach. However, in the context of cancer gene therapy, its utility in the majority of patients in clinical trials was much limited due to the transient expression of the therapeutic gene and its severe

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side-effects.

Concurrent with the above developments, increasing knowledge concerning the importance of dendritic cells (DCs) in generating immune responses has stimulated attempts to use DCs in cancer vaccines [3]. Recently, immunization with either tumor antigen-presenting DCs or genetically modified DCs has been reported in animal models and clinical trials [4, 5]. However, the antitumor effect of monotherapy using either gene therapy or DC vaccine against established tumors is still far away from satisfactory. On the other hand, combined immunotherapy using both the above-said modalities may become an effective strategy in the implementation of future clinical objectives.

Gene therapy

Gene therapy is regarded as transfer of therapeutic genetic material into cells for the purpose of treating or eliminating the causes of a particular disease. Currently, cancer gene therapy mainly uses two types of gene-delivery vectors, namely viral and the non-viral vectors. [6].

The non-viral vectors include naked plasmid DNA, DNA complexed with cationic lipids, and particles comprising DNA condensed with cationic polymers. The advantages of these vectors over their viral counterparts are that they: (i) have no limitation with regard to insert-size, (ii) are less immunogenic, and (iii) are easier to produce. However, a major drawback of non-viral vectors is that their *in vivo* transfection is not as efficient as those of viral vectors. For this reason, viruses are increasingly being developed as vectors for gene delivery.

Vectors based on retroviruses or AdVs have been used most frequently in cancer gene therapy [7]. Retroviruses integrate their genome into host DNA, and this provides the possibility of long-term transgene expression. However, this random integration may also render the retrovirus vector the potential for insertional mutagenesis in host cells. Retrovirus vectors only transduce dividing cells. Because most cells *in vivo* are quiescent, the use of retrovirus vectors in cancer gene therapy trials is limited to manipulation of tumor cells *ex vivo*.

Since their isolation in 1954 [8], AdVs have become a model system for studying gene expression and regulation, DNA replication, regulation of apoptosis, and virus-host interactions [9]. Under natural conditions, AdVs are usually associated with minor human diseases such as upper respiratory tract infections, keratoconjunctivitis and gastroenteritis. Under experimental conditions, early clinical vaccination with wild-type (wt) live AdVs showed no significant side effects, demonstrating their relative safety as vectors for *in vivo* gene therapy. In recent years, AdVs have been increasingly considered as gene therapy vectors for treating human diseases for the following features: (i) they have been proven safe and effective after being used as live vaccines for immunizing millions of military recruits against acute respiratory infections [10]; (ii) they do not integrate their viral DNA into host chromosomes, thereby avoiding the possibility of disturbing vital cellular genes or inducing cancer as has been the case with retrovirus vectors [11]; (iii) they can modulate dendritic cell maturation by increasing the expression of major histocompatibility complex (MHC) and costimulating molecules [12]; (iv) they can accommodate a large size of foreign DNA of up to 37 kb; and (v) they can be easily produced in large quantities.

To date, several strategies of AdVs-mediated gene therapy have been developed for cancer treatment. They include: (i) AdVs-mediated cytokine or immune accessory molecule gene therapy, (ii) AdVs-mediated tumor suppressor gene therapy and (iii) AdVs-mediated chemogene therapy.

Adenovirus-mediated cytokine or immune accessory molecule gene therapy

Cytokines such as interleukin-2 (IL-2), gamma interferon (IFN-γ) and alpha tumor necrosis factor (TNF-α) are important mediators of immune responses against cancer [13-15]. However, the systemic use of these cytokines in cancer patients is limited because of the low concentrations of cytokines in tumors and the severe toxic side-effects derived from high-dose cytokine administration. To improve therapeutic efficacy, cytokine genes have been introduced into tumor cells on the premise that if they can be locally secreted, this would circumvent the limitations associated with their systemic administration.

The foregoing concepts will be highlighted by a number of studies. For example, there are reports documenting the success of *in vivo* cancer gene therapy in animal models with recombinant AdVs vectors expressing IL-2 [16, 17], IL-6 [18], IFN-γ [19, 20] and TNF-α [21]. Significant regression of pre-existing tumors has been observed in these studies, indicating that AdVs vectors expressing cytokines could potentially form the basis for highly effective cancer gene therapy. However, one of problems observed when using AdVs-mediated cytokine gene therapy is the rapid decline in the cytokine expression within the tumors. This may be due to the local diffusion or absorption of the recombinant AdVs through the vascular system. Therefore, repeated injections of recombinant AdVs in high titers is usually required in order to maintain a high local concentration of the cytokines. However, severe toxicity resulting from vaccination of AdVs expressing cytokines with too high titers has also been reported [17].

Combinational cytokine gene therapy has been used to boost the anti-tumor immunity and at the same time to

minimize the side-effects of cytokines. IL-12 mediates potent anticancer effects by induction of cytotoxic T lymphocytes (CTLs), Th1-type immune responses, activation of natural killer (NK) cells [22], and by impairment of tumor vascularization [23]. However, the use of IL-12 for the treatment of cancer patients was unsuccessful because of unacceptable dose-related toxicity, which, in some instances, even resulted in fatalities [24]. To overcome this obstacle, Narvaiza and colleagues [25] used a combinational approach which involved the intratumoral (i.t.) coinfection of two AdV vectors encoding IL-12 and interferon-g inducible protein-10 (IP-10) respectively. IP-10 is a chemokine that is able to recruit T and NK cells to the tumor cells and impair tumoral angiogenesis [26, 27]. In a mouse colorectal adenocarcinoma model, i.t. injection of AdV vector expressing IL-12 (AdVIL-12) induced tumor regression in nearly 70% of cases. However, when a suboptimal dose of AdVIL-12 was co-injected with the AdV vector expressing IP-10, 100% tumor eradication was achieved not only in the locally injected region, but also in distant non-injected tumor sites.

The costimulatory molecule B7-1, which is critical for the generation of T cell mediated immunity, is also used with IL-12 for combinational treatment of established mouse mammary adenocarcinoma. The AdV vector was constructed by insertion of the IL-12 gene and B7-1 gene into E1 and E3 regions respectively [28]. A single intratumoral (i.t.) injection of the virus expressing both B7-1 and IL-12 at 2.3×10^7 plaque forming units (pfu) per mouse resulted in complete tumor regression in 70% of treated animals, compared to only 30% for animals injected with a virus expressing either IL-12 or B7-1. In addition, the cured animals remained tumor free after rechallenge with fresh tumor cells, indicating the presence of a protective immune memory response induced by the combinational treatment. A similar strategy was also used to express lymphotactin and IL-2, or lymphotactin and IL-12, in a single AdV vector [29]. The i.t. injection of these constructs significantly enhanced antitumor immune responses in mouse breast cancer models.

Adenovirus-mediated tumor suppressor gene therapy

Since most human cancers arise from the loss or mutation of regulatory components in cell-cycle-controlling pathways [30], a major strategy for cancer gene therapy has been developed by restoration of tumor suppressors in cancer cells [31]. For example, mutations in p53 are the most common genetic alterations in cancer cells [32]. In addition, p53 is a potent inducer for triggering apoptosis, and is effective despite the presence of multiple genetic alterations in the cancer cells [33]. Thus, AdV-mediated p53 transfer (AdVsp53) is extensively used for cancer gene therapy. Mutation of the p53 tumor suppressor gene contributes to the progression of human prostate cancer. A mouse model for human prostate cancer was used to study whether introduction of AdVsp53 had any impact on primary tumor growth as well as progression to metastatic disease [34]. Infection of human prostate cancer cells *in vitro* with AdVsp53 resulted in marked growth inhibition and apoptosis. *In vivo* studies demonstrated that a single injection of AdVsp53 into an established prostate tumor resulted not only in primary tumor growth suppression, but also reduced the frequency of progression to metastatic disease. These results suggest that an AdVsp53 gene therapy strategy may prove useful in the treatment of human prostate cancer. The efficacy of AdVsp53 cancer gene therapy has also been demonstrated in other studies [35], and other types of tumors such as breast [28] and brain cancers [36, 37].

Although p53 appears to be an appealing target for cancer gene therapy, other genes involved in the inhibition of cyclin-dependent kinases have been explored for induction of apoptosis in tumor cells. $p16^{INK4A}$, which functions as a negative cell-cycle regulator by controlling the activity of CDK4-cyclin D [38], is frequently deleted, mutated, or silenced by promoter methylation in many human cancers [39]. Therefore, restoration of the $p16^{INK4A}$ gene in p16-depleted tumor cells would significantly inhibit tumor growth. Based on this concept, AdV-mediated $p16^{INK4A}$ gene transfer as a cancer gene therapy approach was studied in several types of human cancers with $p16^{INK4A}$ deletion, including non-small cell lung cancer, esophageal, prostatic, pancreatic and breast cancers [40-42].

A combined delivery of two tumor suppressor genes has been used to enhance therapeutic efficiency. Sandig and colleagues [43] treated hepatocellular carcinoma by coexpression of $p16^{INK4A}$ and p53 with AdV vectors. They demonstrated that overexpression of $p16^{INK4A}$ results in a blockage of cell division and, subsequently, in a gradual reduction of the levels of Rb whereas overexpression of both $p16^{INK4A}$ and p53 induces apoptosis in tumor cells. In a mouse hepatocellular carcinoma model, simultaneous AdV transfer of p16 and p53 genes further leads to inhibition of tumor growth in nude mice. Shinoura and colleagues [44] further demonstrated that AdV-mediated transfer of p53 and $p33^{ING3}$, which cooperates with p53 to block cell proliferation, drastically augments apoptosis in gliomas. Therefore, combined delivery of two cooperating tumor suppressor genes could be the basis for the development of a new strategy for cancer gene therapy.

Adenovirus-Mediated Chemogene Therapy

In an attempt to avoid side-effects of conventional chemotherapy, AdV vectors have been used to transduce

tumor cells with the chemogenes encoding an enzyme that converts a nontoxic substance (prodrug) into a toxic molecule. Therefore, chemogene therapy is also called the enzyme/prodrug approach. Since the enzyme encoded by chemogenes is not normally present in human cells, toxins should selectively kill tumor cells transduced by the chemogene as well as the surrounding untransduced cells through a bystander effect.

A variety of genes encoding different types of enzymes have been investigated for their potential use in cancer gene therapy. The most commonly used chemogenes for treatment of various cancers is Herpes Simplex virus thymidine kinase (HSV-TK) with the prodrug gancyclovir (GCV) [45]. The drug GCV is nontoxic as long as it is not metabolized. It is a poor substrate for human TK but is metabolized to monophosphate GCV (MP-GCV) by HSV-TK. Subsequently, cellular enzymes convert MP-GCV to triphosphate-GCV, which is incorporated into DNA and RNA. This confers cytotoxicity and cell death by termination of DNA and RNA synthesis. Therefore, tumor cells expressing HSV-TK may be selectively killed by infusion of GCV.

Many articles have documented the utility of i.t. injection of AdVs expressing HSV-TK genes in animal models [46-48]. Having demonstrated a prolonged survival in the murine ovarian cancer model treated with AdV-mediated HSV-TK transfer [49], Alvarez and colleagues [50] further conducted a phase I study to determine the efficacy of this novel approach in patients with recurrent ovarian cancer. Fourteen patients were enrolled in the study. In contrast to brain tumor injections, no dose-limiting toxicity was observed with the intraperitoneal administration of an AdV vector expressing HSV-TK at the dosages studied. The peritoneal cavity thus appears to be fairly tolerant of AdV vector treatment compared to other closed compartments. Of the thirteen patients evaluated, five had stable disease and eight had evidence of progressive disease. The presence of HSV-TK transgene was demonstrated in most patients two days after AdV vector administration. These results suggest that HSV-TK chemogene therapy is feasible for the treatment of intraperitoneal neoplastic conditions such as metastatic ovarian cancer. Phase I clinical trials using AdV-mediated HSV-TK transfer have also been completed for other types of cancer including malignant mesothelioma, prostate cancer, and malignant brain tumors [51, 52].

Another widely used chemogene is the bacterial and fungal gene encoding cytosine deaminase (CD), which deaminates cytosine to uracil and 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU). Due to the lack of CD in mammalian cells, the CD gene has become an attractive candidate for chemogene cancer therapy. Transfer of the CD gene to tumor cells allows conversion of relatively non-toxic 5-FC to 5-FU. The latter inhibits both RNA and DNA synthesis and leads to cell death. AdV-mediated CD gene transfer has been used in gene therapy for various cancers including colon, prostate, and cervical carcinomas [53, 54]. Adachi and colleagues [55] attempted combinational chemogene therapy for brain tumors. They introduced both the CD gene and the uracil phosphoribosyltransferase (UPRT) gene into rat 9L gliosarcoma cells using AdV vector, thus encoding the enzyme to convert 5-FU to 5-fluoroluridine 5'-monophosphaste. Coexpression of CD and UPRT renders 9L cells 6 000 times more sensitive to 5-FC than the CD gene expression alone. This approach also significantly prolonged animal survival in a rat brain tumor model. Similarly, the CD gene has also been coexpressed with cytokine genes to increase the cytotoxicity of 5-FC [56].

In the last decade, much progress has been made in AdV-mediated gene therapy of cancer. However, a number of significant problems still confound this treatment modality, namely (i) the failure to efficiently infect certain primary tumor cells which lack AdV receptors such as the Coxackie adenovirus receptor (CAR), and (ii) the promiscuous tropism which causes uncontrolled gene transfer in normal bystander cells. Over and above this, the immune responses associated with gene therapy may lead to other problems such as i) the transient expression of therapeutic gene, ii) non-efficient re-administration of the same vectors, and iii) severe side-effects as has been documented in clinical trials. In an attempt to circumvent or lessen these challenges, various cancer immunotherapeutic strategies are being devised. One such strategy that deserves mention is the DC-based tumor vaccine.

Dendritic cell-based tumor vaccines

Mature DCs (mDCs) are characterized by having numerous membrane processes that take the form of dendrites, pseudopods, or veils. As the most potent antigen-presenting cells (APC) for primary immune responses, they are also characterized by displaying high levels of MHC class II antigens, and various adhesion and costimulatory molecules (e.g., CD11a, CD11b, CD11c and CD54) on their surface. As with other APCs, the costimulation-associated molecules CD80, CD86, and CD40 are expressed on mDCs, and CD83 is now also recognized as a specific marker for human mDCs. Dendritic cells can process antigens via the classical pathway, whereby endogenous antigens are delivered via proteosomes into the MHC class I compartment, and exogenous antigen via endocytic lysosomes into the MHC class II compartment. However, they also possess an alternative pathway of antigen processing whereby they route exogenous antigen into the MHC class I pathway through a mechanism known as cross-priming. They can

also utilize molecular chaperones, such as heat shock proteins (HSP), to deliver antigens via the class I pathway [4]. Murine splenic DCs can express CD4 or CD8 markers. It is known that various subsets of DCs exist in humans and mice and that they can play differing roles in the regulation of immune responses [57, 58]. DCs have also been shown to be capable of inducing strong antitumor immunity [3-5].

Based upon the availability of recombinant cytokines essential for DCs growth and maturation, bone marrow-derived DCs (BM-DCs) can now be generated in large numbers simply by culturing BM cells in granulocyte monocyte-colony stimulating factor (GM-CSF) and IL-4 [59]. However, the phenotype of the DCs so generated is critically dependent upon the precise culture conditions. BM cells cultured with low doses of GM-CSF (2 ng/ml) alone or high doses of GM-CSF and IL-4 (each, 10-20 ng/ml) can differentiate into immature DCs (iDCs) or relatively mature DCs (rmDC) respectively [6]. iDCs lack expression of MHC Class II and costimulatory molecules, while rmDC express intermediate levels of MHC class II and CD80 and low levels of CD40. The rmDC can be induced to full cellular maturity (i.e., mDCs) by exposure to stimuli such as LPS, DNA, TNF, HSP or agonistic anti-CD40 antibodies [61-63]; they then express yet higher levels of MHC class II, CD40, CD80, as well as other maturation markers. Injection of Flt3-ligand into humans or mice leads to a substantial expansion of the total DC population [64, 65], while AdV transduction of DC precursors with a GM-CSF gene renders exogenous GM-CSF supplementation unnecessary for the production of mature DCs in culture [66]. Such technical advances, when combined with the increasing knowledge of the important roles DC have in the initiation of immune responses; have provided a compelling impetus for pursuing DC-based immunotherapies for cancer. Here, we briefly summarize recent progress with DC-based cancer vaccines, including tumor antigen-presenting DCs, and genetically-engineered DC vaccines.

Tumor antigen-pulsed DC vaccine

Tumor-antigen-pulsed DCs have been demonstrated to induce the development of MHC class I- and class II-specific T cell responses *in vitro* and *in vivo*. DCs pulsed *in vitro* with peptide antigen and subsequently given to animals can induce antigen-specific, CTL-mediated protection against lethal tumor challenges, and can even induce regression of established tumors [67]. Delivery of the same peptide by DCs can lead to dramatic immunostimulation, while administration of tumor peptides alone can lead to peptide-specific CTL tolerance [68]. Delivery of an array of human and mouse tumor antigens/peptides by DCs can lead to marked antitumor immune responses. Specifically, MHC-restricted synthetic tumor-associated peptides such as melanoma-related antigen, endogenous retroviral gene products gp70/p15E, carcinoembryonic antigen (CEA), folate binding protein (FBP), prostate-specific membrane antigen (PMSA), survivin, MUC-1, HER2/neu and idiotypic protein (Id)-derived peptides, synthetic *bcr-abl* b3a2 fusion peptide (ATGFKQSSKALQRPVAS) and a synthetic HPV 16 E7 peptide have been identified. The tumor associated peptides have been delivered to DCs, and used with various degrees of success in animal studies or clinical trials [69-76].

Vaccination strategies directed against a single tumor antigen peptide or epitope may be unduly narrow in scope, with the immune system investing all of its effector resources in a 'single' response. On the other hand, the use of whole tumor lysates as a source of antigen offers the potential advantage of inducing broad-spectrum T cell responses against multiple known, as well perhaps hitherto unknown, tumor-associated antigens expressed within the tumor. As illustrated above, these might induce not only the critical baseline CTL responses, but also helper T cell responses important for a more complete realization of the full antitumor CTL potential. This approach also reduces the work required to identify and generate individual peptides. In two separate strains of mice with histologically distinct tumors, the subcutaneous (s.c.) injection of tumor lysate-pulsed DCs has been shown to effectively prime them for subsequent rejection of lethal challenges with viable parental tumor cells, and also to significantly reduce the number of metastases subsequently established in the lungs of these animals [77]. Furthermore, experimental tumor lysate-DC vaccination has been shown to be effective against hepatocellular carcinoma BNL1MEA.7R.1 (BNL), murine renal cell carcinoma, and syngeneic GL261 gliomas in mice [78, 79]. Similarly, DCs that have been pulsed with lysates from pancreatic carcinoma cells, malignant brain tumors, or ovarian cancers can induce tumor antigen-specific CTL responses *in vitro* [80, 81]. Most importantly, DC-tumor lysate vaccination of cancer patients has also been shown to be beneficial in the treatment of malignant melanoma, parathyroid carcinoma, advanced breast and uterine cancer, renal cell carcinoma (RCC) and solid tumors [82-86].

Tumor mRNA-pulsed or transfected DC vaccines

Just as DCs can be treated with tumor peptides or lysates, they can also be pulsed or transfected with tumor RNA. Successfully transfected DCs then translate the respective tumor proteins, with all of the epitopes they encode. After being processed, these tumor antigens would also have the advantage of possessing broader HLA specificities and thus permit the induction of CTL responses almost irrespective of the patient's HLA repertoire. A further advantage of using mRNA is that it can be isolated from murine tumor cell

lines or from primary human tumor cells microdissected from frozen tissue sections, and amplified at will without loss of function [87, 88].

DCs transfected with tumor cell mRNA can stimulate potent CTL responses and engender protective immunity in tumor-bearing mice [87]. Tumor mRNA can be efficiently transfected into DCs, resulting in superior translation product yields in these cells relative to other professional APCs. Most researchers have used mRNA/liposome complexes to transfect DCs, although more efficient mRNA delivery may be achieved by electroporation when using human hematopoietic cells [89, 90]. Such mRNA-mediated delivery of encoded tumor antigens to DCs can induce potent primary T cell responses *in vitro*. This is largely because transfection of DCs with tumor mRNA delivers maturation/activation signals to the cells and mediates efficient delivery of antigenic peptides to MHC class I and II molecules. Thus, when used in anti-tumor vaccine strategies, this approach has the potential to powerfully induce tumor-specific effector T cell activation [91, 92]. Investigations such as these provide a theoretical foundation for broadly applicable tumor treatments that do not require prior characterization of the relevant antigenic profile for each patient (i.e., the tumor peptides presented by their own HLA haplotype specificities) and would not be limited by the availability of tumor tissues for antigen preparation [88]. Although the total tumor RNA-transfected DC vaccines are still limited in clinical trials [93, 94], it may represent a broadly applicable vaccine strategy to induce potentially therapeutic polyclonal T-cell responses in cancer patients.

Necrotic or apoptotic tumor cell-loaded DC vaccines

DCs can readily take up soluble tumor antigens, such as proteins or immune complexes, but can also phagocytose dying (e.g., apoptotic or necrotic) tumor cells, and thereby induce protective antitumor immunity [95, 96]. The recognition and uptake of apoptotic cells by DCs is regulated by specific receptors such as aVb5, CD36, or the phosphatidylserine receptor [97], while uptake of necrotic cells is mediated by CD91, the receptor for HSP exposed on these cells [98, 99]. The advantages of using dying tumor cells as a source of tumor antigens are that: (i) DCs can present or cross-present both MHC class I and II epitopes of a defined tumor antigen or multiple tumor antigens (e.g., MAGE3 and gp100 of melanoma tumors) [100-102]; and (ii) unlike the case with peptide-pulsed DCs, this approach is independent of HLA haplotype and can thus be applied equally to all patients.

The uptake of dying cells decidedly impacts DC maturation. According to the "danger signal" theory of Matzinger [103], the immune system should be activated by internal injuries that signal threats to the organism such as cellular necrosis, but not by signals associated with more "normal" homeostatic processes, such as apoptosis [104]. In this context, it is interesting to note that some reports document that DCs that have captured apoptotic tumor cells induce immunological tolerance to the tumors [105], while other reports indicate that DC phagocytosis of apoptotic tumor cells can also induce effective antitumor immunity [106, 107]. It has now been clearly demonstrated that the stage of the target cell within the apoptotic process affects the maturation of DCs engulfing the cells and thus also the antitumor immunity these cells can induce. Specifically, only tumor cells in the late, but not early, phases of apoptosis stimulate DC maturation and antitumor immunity [108]. Recent comparative analyses have shown that necrotic and late phase-apoptotic cells equally trigger DC maturational changes that lead to the induction of antitumor immunity [109]. As noted above, DC phagocytosis of necrotic tumor cells is dependent on their expression of HSP, as is their subsequent maturation and ability to induce anti-tumor immunity [109-111]. Our results also showed that DCs phagocytosed necrotic/apoptotic tumor cells (as a result of exposure to lovastatin) undergo strong maturation responses, with up-regulated expression of proinflammatory chemokines and cytokines, and co-immunostimulatory molecules. These cells induce stronger protective immunity against tumor challenge in animal models than do DCs pulsed with MHC class I-restricted tumor peptides [112]. Thus, DCs that have phagocytosed apoptotic/necrotic tumor cells appear to offer another new strategy in DC cancer vaccination.

Gene-modified DC vaccines

Although the approaches described above are encouraging, they are less likely to be applied in the majority of clinical cases largely due to the technical difficulties inherent in the preparation of such materials from human solid tumors. A new strategy, employing genetically modified DCs, has recently been developed for use in DC vaccination. The target genes transferred into the DCs fall into two categories, tumor associated antigens (TAA) and immunomodulatory proteins such as cytokines or co-stimulatory molecules. Various methods have been used to introduce genes into DCs, including cationic lipids, electroporation, biolistic delivery (i.e., the "gene gun"), complexes of plasmid DNA expression constructs with the cationic peptide CL22, nonviral T7 vector, viral vectors and AdVs/polycation complexes [113-116]. The viral vectors that have been used up to now are poxvirus such as modified vaccinia Ankara (MVA), retrovirus as exemplified by the new lentiviral vectors derived from SIVmac251 (a simian immunodeficiency virus (SIV), herpesvirus, Semliki Forest virus (SFV), influenza virosomes, adeno-associated

virus, canarypox virus and AdVs [117-120]. From amongst them, the AdV vector was deemed to be a good candidate because of its high efficiency and the attendent minimal risk associated with insertional mutagenesis.

DCs engineered to express tumor-associated antigens The strategy of using genetically modified DCs expressing specific cancer antigens has several advantages over using DCs simply pulsed with tumor antigen proteins or peptides. These include (i) a reduced need to assess the immunologic relevance of individual cancer-specific peptides (as long as the molecules transduced into the DCs are immunogenic), and (ii) the tumor proteins being constitutively-synthesized within the DC will permit specific antigen presentation to T cells for longer periods without generating concerns about the breakdown of peptide/MHC complexes. DCs that express tumor antigen transgenes are also more potent primers of antitumor immune response than their soluble antigen-pulsed counterparts, as determined both in vitro and in animal models [121]. Another advantage of using DCs engineered to express tumor antigens is their potential for generating $CD8^+$ T cell responses against multiple class I-restricted epitopes within the antigen, thereby eliciting a broad antitumor effector response [119]. Immunization through ex vivo transduction of DCs has been demonstrated as an effective approach to enhance antitumor immunity by activating $CD8^+$ T cells [122]. MAGE-1, gp100, MART-1, hTRP2, p53, MUC-1 and other antigen genes have been used to transfect murine and/or human DCs thereby inducing tumor antigen-specific immune responses [121, 123-127].

Replication-deficient recombinant AdVs encoding human gp100 or MART-1 melanoma antigen have been used to transduce human DCs *ex vivo* in model systems for cancer vaccine therapy. Human DCs that have been transduced with a replication-defective E1-deleted AdVMART1 produce full-length MART-1 mRNA and protein. *In vitro* challenges with such DCs stimulated MART-$1_{(27-35)}$-specific tumor-infiltrating lymphocytes to synthesize IFN-γ and induced the generation of peptide-specific, MHC class I-restricted CTL within peripheral blood lymphocyte (PBL) from normal donors. A second generation E1/E4 region-deleted AdV (which harbors the CMV immediate-early promoter/enhancer and a unique E4-ORF6/pIX chimeric gene; Ad2) has also been developed. DC transduced with Ad2/gp100V2 can elicit tumor-specific CTL *in vitro* from patients bearing $gp100^+$ metastatic melanoma [128]. Similarly, transduction of an HLA-$A2^+$/MART-1- cell line with AdVMART1 renders these cells sensitive to lysis by CTL specific for the MART-$1_{(27-35)}$ immunodominant peptide [129]. Mice vaccinated with AdVMART1-DCs generated protective responses to lethal tumor challenges with murine B16 melanoma cells. These responses were mediated by MHC class I-restricted, MART-1-specific CTLs which produce high levels of IFN-g when re-exposed to MART-1 *in vitro,* and kill their targets in a manner suggestive of perforin/granzyme-dependent lysis [124].

Genetic immunization using DCs transduced *ex vivo* with an AdV expressing the HER2/neu gene (AdVNeu) can also induce immunity against a breast tumor cell line overexpressing HER2/neu [130]. Subcutaneous immunization with this DC vaccine elicited protective immunity from tumor challenge in 60% of the treated animals, and CTL analyses demonstrated that the animals displayed specific cytotoxic activity against breast tumor cells, as well as syngeneic fibroblasts transduced with AdVNeu. *In vivo* depletion studies demonstrated that, here too, both $CD4^+$ and $CD8^+$ T cells were required for effective immunity. In a therapeutic setting, these immunizations could cure mice with established tumors, with the efficacy of this effect being further enhanced by transducing the DCs to express murine IL-12 (AdVmIL-12) [130]. Autologous $CD34^+$ hematopoietic progenitor-derived DC retrovirally-transduced with a HER2/neu gene elicited HER2/neu-specific $CD8^+$ CTLs that lyse HER2/neu-overexpressing tumor cells in the context of distinct HLA class I alleles. The induction of both HLA-A2 and -A3-restricted HER2/neu-specific CTLs was verified at the clonal level, and the presence of $CD4^+$ Th1 cells recognizing HER2/neu in the context of HLA class II was also documented. These HLA-DR-restricted $CD4^+$ T cells were cloned and found to release IFN-g upon stimulation with DCs that had been pulsed with HER2/neu extracellular domain. These data indicate that retrovirally-transduced DCs expressing the HER2/neu molecule present multiple peptide epitopes and elicit HER2/neu-specific CTL and Th1 cells. More importantly, this method of stimulating HER2/neu-specific $CD8^+$ and $CD4^+$ T cells with retrovirally-transduced DCs could also be successfully employed for *in vitro* generation of HER2/neu-specific CTL and Th1 clones from patients with HER2/neu-overexpressing breast cancers. From a conceptual and practical viewpoint, this is a significant advance in the field of DC vaccination therapy since it provides a method for the generation and expansion of HER2/neu-specific, HLA-restricted CTL and Th1 clones *in vitro*. This should facilitate effective adoptive transfer of autologous HER2/neu-specific T cell clones into patients with HER2/neu-overexpressing tumors without the need to define each tumor's immunogenic peptides [131].

DCs engineered to express immunomodulatory molecules As outlined in detail above, transduction of DCs with tumor antigens offers distinct advantages over simple pulsing of the cells with tumor peptides or lysates. Nevertheless, a notable disadvantage is the painstaking task of selecting an

appropriate tumor antigen as the DC transduction candidate. MHC haplotype restrictions apply in the presentation of CTL and other epitopes, such that a substantial proportion of the candidate patient base may not be capable of responding to any one chosen peptide/antigen. An alternate strategy has been developed recently to augment the ability of DCs to present tumor antigens, namely transducing them with expression vectors such that they constitutively express immunomodulatory proteins such as cytokines and chemokines. Thus, DCs genetically modified to express a T cell stimulatory cytokine, for example, could possess adjuvant-like properties useful in the treatment of any number of tumors, so long as sources of TAA were available. It can be argued that one could transduce the tumor cells themselves instead, and count on their subsequent recruitment of APCs. However, since DCs are by nature professional APC designed to deliver their cytokines in precisely the correct context [132], immunomodulatory gene-modified DCs (GM-CSF, TNF-α, IL-12, SLC, lymphotactin and CD40L etc.) would represent potentially more potent vaccines than similarly modified tumor cells [133-138].

GM-CSF is an essential *in vitro* growth and differentiation factor for DCs [59]. The fact that *in vivo* administration of GM-CSF augments primary immune responses suggests that enforced GM-CSF expression by DCs could perhaps further enhance the effectiveness of DC-based immunotherapy protocols. *In vitro*, the phenotype of BM-DCs remains largely unaltered by GM-CSF gene transfection, but infection of the DC cell lines XS52-4D and XS106 with AdV-GM-CSF upregulates their expression of MHC and costimulatory molecules as well as their alloantigen or peptide antigen-presenting capacities. On the other hand, when used for *in vivo* immunizations, the antigen-presenting capacity of GM-CSF gene-transfected BM-DCs was greatly enhanced relative to mock-transfected or untreated DC, as determined by their ability to induce primary immune responses to haptens, protein antigens, or tumor antigens. This increased efficacy correlated with an augmented migratory capacity of GM-CSF gene-transfected BM-DCs *in vivo*. These data thus suggest that GM-CSF gene transfection may be useful in improving DC-based vaccines currently under clinical investigation [133, 139].

IL-12 is a heterodimeric cytokine produced by many types of cells, including DCs, macrophages, leukocytes, and keratinocytes [140]. It can enhance NK cell and CTL activities, and plays a key role in the induction of Th1-type immune responses. DCs expressing an IL-12 transgene can promote enhanced specific anti-tumor CTL responses compared to nontransduced DC [135]. Similarly, i.t. injection of such IL-12 transduced BM-DCs leads to regression of weakly immunogenic (day 7) established tumors (MCA205, B16, and D122) and to complete regression of established murine transplantable colon adenocarcinomas. This DCIL-12 antitumor effect (and the induction of tumor-specific Th1 responses) is substantially greater than that observed with similarly IL-12-transduced syngeneic fibroblasts or nontransduced BM-DCs. Splenic DCs engineered to express augmented levels of IL-12 also elicit therapeutic antitumor immune responses [141, 142].

Secondary lymphoid tissue chemokine (SLC) is a CC chemokine that is selective in its recruitment of naive T cells and DCs [143]. In the lymph node, SLC is believed to play an important role in the initiation of immune responses by co-localizing naive T cells with DCs that are presenting (tumor) antigen. i.t. injection of SLC-expressing DCs (DC_{SLC}) results in tumor growth inhibition that is significantly better than observed with either control DCs or SLC alone. Similarly, distant site immunization of tumor-bearing mice with DC_{SLC} that have been pulsed with tumor lysates elicits antitumor responses, whereas controls DCs do not. Direct administration of DC_{SLC} into growing B16 melanomas induces a substantial and sustained influx of T cells into the tumor mass, and there is only transient increase in T cell numbers in the draining lymph node (DLN). This suggests that the DCs are largely retained at the tumor sites, with only a very small proportion of them trafficking to the DLN. Within 24 hours, the T cells infiltrating the tumors express the activation marker CD25, and by 7 days they will have developed an IFN-γ-secreting function, in concert with a detectable inhibition of tumor growth. These reports demonstrate that SLC expression by DCs can induce antitumor responses that lead to enhanced antitumor immunity [136].

Combined AdV-mediated cancer gene therapy and DC-based tumor vaccines

To date, the therapeutic efficiency of DC vaccine strategies remains limited to the generation of prophylactic antitumor immunity against re-challenge with the parental tumor cells. In clinical practice, most candidates for cancer therapy are patients with a sizable tumor load. Therefore, immunotherapeutic strategies aimed at directly combating well established tumors as seen in real clinical situations are of value in translational research. Unfortunately, at present, most of the current mono-immunotherapeutic protocols, such as AdV-mediated gene therapy or DC-based tumor vaccines, have failed in combating well-established tumors in animal models. This lack of success is likely related to a combination of events, including the somewhat inefficient antitumor immune responses induced by the therapy, and the tumor growth rapidly out-stripping the ability of the patient's immune system to effectively deal with it [144, 145]. Thus, a major strategic goal of current cancer

immunotherapy has become the development of novel therapeutic strategies combating well-established tumors. A combined immunotherapy including gene therapy and DC vaccines would have some advantages over each modality administered as a monotherapy. Recently, our lab has focused on induction of antitumor immunity by combined immunotherapy including DC vaccines and AdV-mediated cytokines gene therapy such as TNF-α, CD40L etc [146, 147].

Combination of AdVTNF-α and engineered DC vaccine efficiently eradicate well-established melanoma

Substantial evidence has now proven that TNF-α is a multifunctional and immunoregulatory cytokine that has antitumor activity, evokes apoptosis, up-regulates adhesion molecules, and activates immune cells such as macrophages, DCs, neutrophils and T cells [148]. Since its antitumor properties have been demonstrated in a variety of in vivo experimental models, TNF-α has attracted attention as a potential antitumor reagent [149-151]. Unfortunately, the systemic administration of recombinant TNF-α protein in human cancer clinical trails has been limited by the problem of dose-related toxicity since the maximal tolerated dose of TNF-α in humans is 40-fold less compared to mice on a weight-for-weight basis [152]. Severe side effects are therefore commonly encountered in cancer patients receiving TNF-α treatment. These include a septic-like shock syndrome, hypotension, malaise, diarrhea, leukothrombopenia, and anorexia. [152-154]. To induce a high local concentration of TNF-α while limiting the systemic use of high dose TNF-α, a technique utilizing isolated limb perfusion with TNF-α has been applied [155].

Gene therapy by using both *in vitro* and *in vivo* gene transfer strategies provides a useful means for the local transgene expression of TNF-α within tumors. Tumor cells transduced with the TNF-α gene *in vitro* displayed loss of their tumorigenicity, and vaccines based on these engineered tumor cells can inhibit tumor growth when re-challenging parental tumors in immunized mice by activated immune responses [156, 157]. However, these *in vitro* strategies are cumbersome to use in humans due to the critical requirements of culturing the autologous tumor cells from patient's neoplasm as well as genetically modifying tumor cells with the TNF-α gene prior to its administration. An alternative is the direct *in vivo* gene delivery mediated by AdVs expressing TNF-α. It has been reported that the i.t. administration of AdVTNF-α resulted in transgene expression of TNF-α in tumors, detection of serum TNF-α within one day to two days, and regression of small palpable tumors within two days to three days in treated mice. However, the well-established experimental tumors did not regress, though their growth was much slowed down [158-160].

The above concepts are borne out in our studies. We developed a novel immunotherapy combined AdVTNF-α-mediated gene therapy with a TNF-α-gene modified DC vaccine ($DC_{TNF-\alpha}$). We went on to investigate its therapeutic efficiency in the treatment of well-established MO4 tumors expressing tumor antigen OVA in animal models, and compared it to either gene therapy or DC vaccine alone [146]. As shown in Figure 1, vaccination of $DC_{TNF-\alpha}$ cells pulsed with the OVA I peptide could stimulate a type 1 immune response with enhanced antitumor CTL activities. While $DC_{TNF-\alpha}$ vaccine could induce protective immunity against challenge of 5×10^5 MO4 tumor cells, and reduce the growth of the small (3-4 mm in diameter), it failed to eradicate any of the large tumors (6-8 mm in diameter). However, it did manage to significantly reduce the rapid tumor growth in the latter group of mice. Almost all tumors showed apparent regression within 5 days subsequent to i.t. injection of AdVTNF-α due to sizable tumor necrosis caused by intravascular thrombosis (Figure 2). However, they rapidly regrew, leading to the death of the mice within approximately another two weeks to three weeks. Our histological data also showed that, invariably, there were residual cancer cells at the edges of necrotic areas of the large tumors (Figure 2), and these are the likely the sources of tumor recurrence. The data from the *in vivo* study showed that AdVTNF-α-mediated gene therapy eradicated small tumors in 6 out of 8 (75%) mice, and the mechanism for this appeared to be massive tumor necrosis. In contrast, none of the large tumors (0%) were destroyed. Interestingly, a combined AdVTNF-α-mediated gene therapy and TNF-α-gene-engineered $DC_{TNF-\alpha}$ vaccine cured 3 out of 8 (38%) mice bearing large MO4 tumors, indicating that the combined immunotherapy strategy is much more efficient in combating well-established tumors than monotherapy of either gene therapy or DC vaccine alone (Figure 3).

Co-administration of iDCs following the AdVCD40L elicits significant regression of established myeloma

CD40 ligand (CD40L) is a 33-kDa type II membrane protein that is a member of TNF gene family. It is preferentially expressed on activated $CD4^+$ T cells [161]. The receptor for CD40L is the CD40 molecule (a member of the TNF receptor family) expressed on APCs, including DCs [162].

The functions of DCs such as Ag capture, Ag presentation and migration have been shown to change dynamically as their maturation progresses [163, 164]. Amongst the multitudinous stimuli for DC maturation, CD40 is one of the most important. In fact, the major $CD4^+$ T cell help signal for DC maturation is provided by the interaction between CD40 expressed on DCs and CD40L on activated

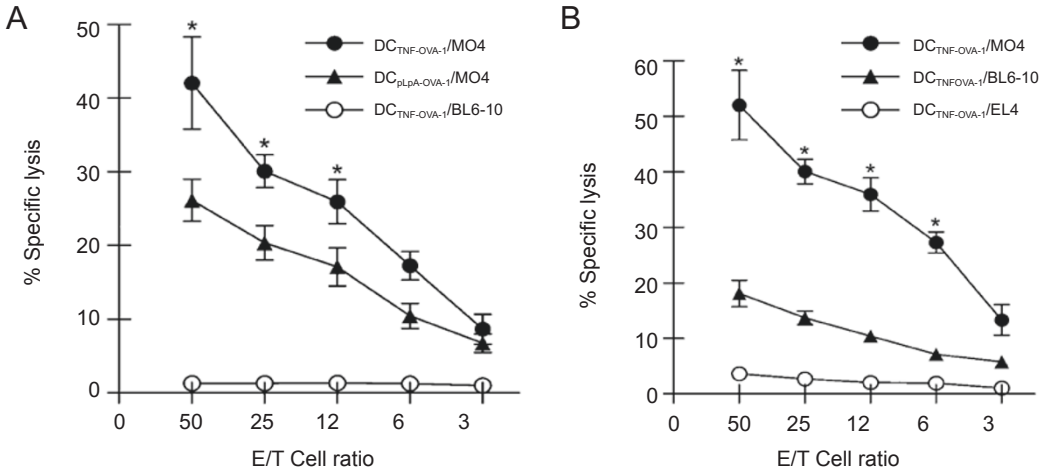

Figure 1 Cytotoxicity assay. **(A)** In the first experiment, splenic lymphocytes from OVA I-pulsed $DC_{TNF-\alpha}$ ($DC_{TNF-\alpha-OVA-I}$) or DC_{pLpA} ($DC_{pLpA-OVA-I}$) vaccinated mice were stimulated *in vitro* for 4 d with irradiated MO4 tumor cells, and then used as effector (E) cells in a chromium release assay. In the assay, ^{51}Cr-labeled MO4 tumor cells with OVA expression were used as target (T) cells. To confirm that the T cell cytotoxicity was MO4-tumor-specific, we also included BL6-10 tumor cells (without OVA expression) as a target control with activated T cells from $DC_{TNF-\alpha}$-vaccinated mice. * $P < 0.05$ versus cohorts immunized with $DC_{pLpA-OVA-I}$. **(B)** In the second experiment, splenic lymphocytes from mice with tumor regression (as a result of treatment with combined gene therapy and DC vaccine) were stimulated *in vitro* for 4 days with irradiated MO4 tumor cells, and then used as effector (E) cells in a chromium release assay. In the assay, ^{51}Cr-labeled MO4, BL6-10 and EL4 tumor cells were used as target (T) cells. * $P < 0.05$ versus cohorts of BL6-10 tumor cells as target cells. Each point represents the mean of three triplicates.

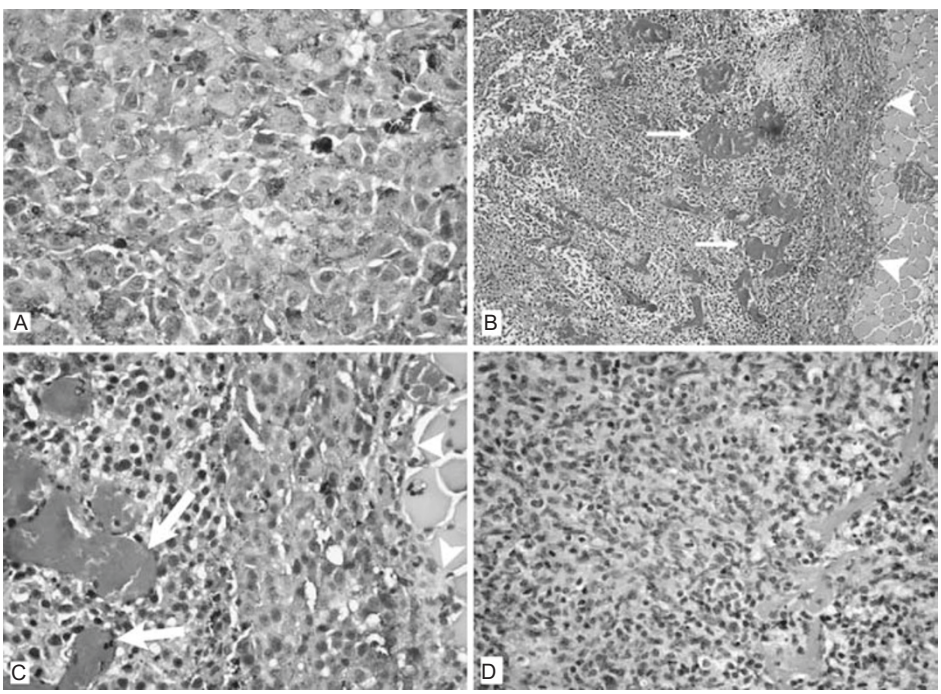

Figure 2 Histologic photomicrographs of tumors and/or lesions. Tumor nodules at the injection sites were removed, fixed in 10% formaldehyde and embedded in paraffin for histological analysis 1 d and 8 d subsequent to the injection of AdVTNF-α. Sections of 6-7 (Jim: 6-7 microns sounds quite thick; it is usually 4-5 microns. But you can leave it like that if it is really 6-7) mm thickness were stained with hematoxylin-eosin according to the standard procedures. **(A)** Photomicrograph from a MO4 tumor showing numerous melanoma cells. **(B, C)** Photomicrograph from a MO4 tumor at d 1 after i.t. injection of 2×10^9 PFU AdVTNF showing extensive tumor necrosis with some residual tumor cells at the edges of the lesion (arrow heads) and intravascular thrombosis (arrows). **(D)** Photomicrograph from a regressed MO4 TS1 tumor at d 8 after the AdVTNF injection displays mostly mononuclear cells and fibroblasts, but no tumor cells. Magnification was × 200 for all except for B which × 100.

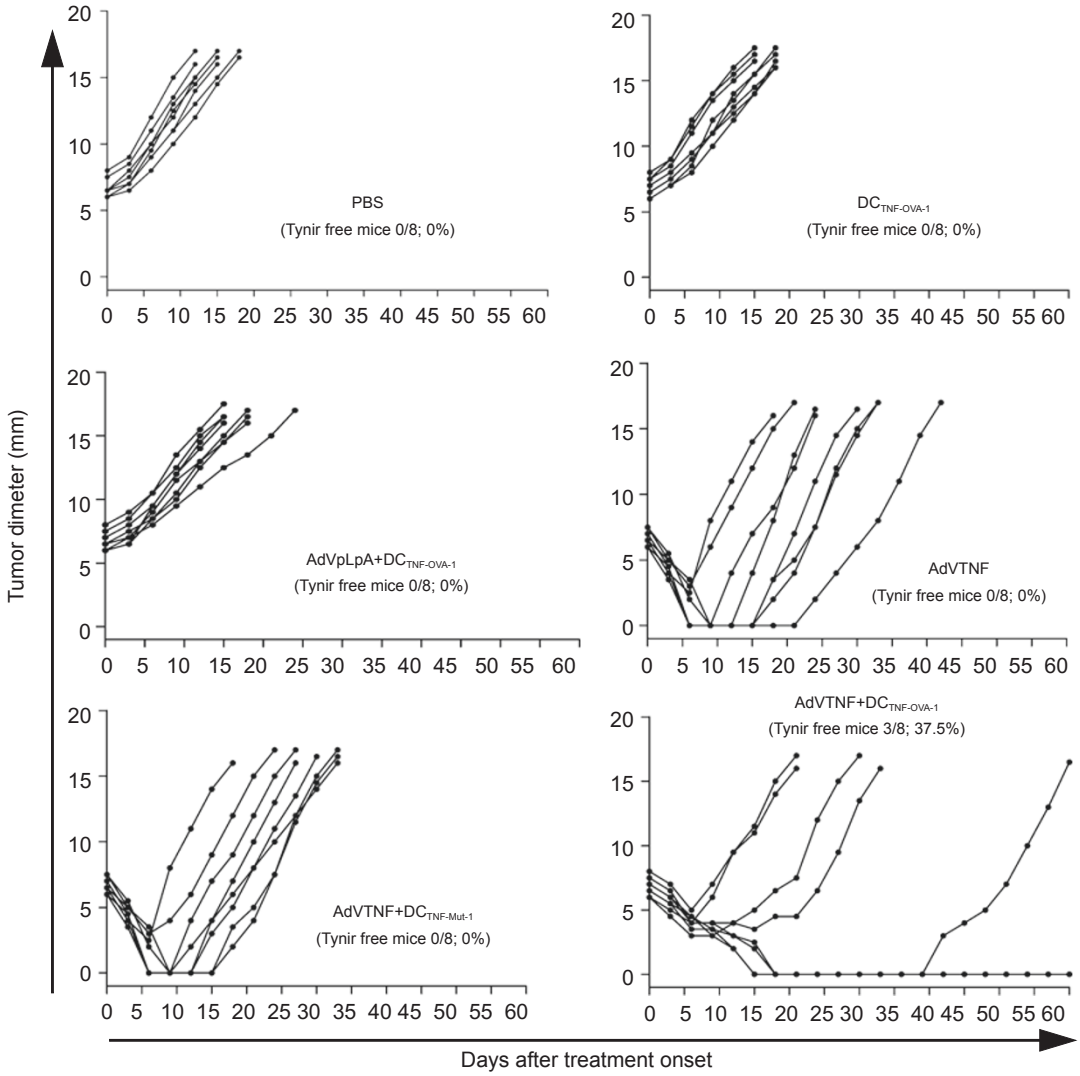

Figure 3 Impact of combined gene therapy and DC vaccine on the growth of well-established tumors in mice. Mice bearing well-established MO4 tumors (6 mm - 8 mm in diameter) were given i.t. injection of AdVTNF-α or AdVpLpA (2×10^9 pfu) in conjunction with subcutaneous OVA I- or Mut 1-pulsed $DC_{TNF\alpha}$ ($DC_{TNF-\alpha-OVA-I}$ or $DC_{TNF-\alpha-Mut-1}$) vaccines three times. Another three control groups of mice were given i.t. injection of PBS or control AdVpLpA alone or subcutaneous $DC_{TNF-\alpha}$ ($DC_{TNF-\alpha-OVA-I}$) vaccines alone. Tumor growth was monitored and the tumor size (diameter) measured using an engineering caliper. The evolution of the tumors in individual mice are depicted, as are the fractions of mice in each treatment group that were tumor-free at 60 days post-treatment.

$CD4^+$ T cells [162, 165]. It has been shown that that mice lacking CD40L were associated with immunodeficiency accompanied by an impaired migration of DCs into secondary lymphoid tissues [166], thus reinforcing the notion that CD40 stimulation is indispensable for DCs maturation. It is well known that, for antitumor immunity to be effective, the $CD8^+$ T cells must recognize tumor Ag-peptides bound to MHC class I expressed on the tumor cells. However, for $CD8^+$ T cells to acquire the ability to destroy the tumor cells, the DCs presenting the Ag must receive the $CD4^+$ T cell help signal [167, 168]. The functions of DCs are mainly dependent on their state of activation and differentiation, i.e., iDCs can avidly capture Ags, while mDCs can efficiently induce CTL cytotoxicity. However, the most optimal method for conditioning DCs for anticancer immunotherapy is not entirely clear. Watanabe S and his colleagues found that iDCs had significant advantages over mDCs in anticancer immunotherapy due to their ability to prime T cells in secondary lymphoid organs through immobilized anti-CD40 antibody stimulation in the *in vitro* model [169]. Therefore, we hypothesized that AdVCD40L i.t. injection might have the ability to inhibit

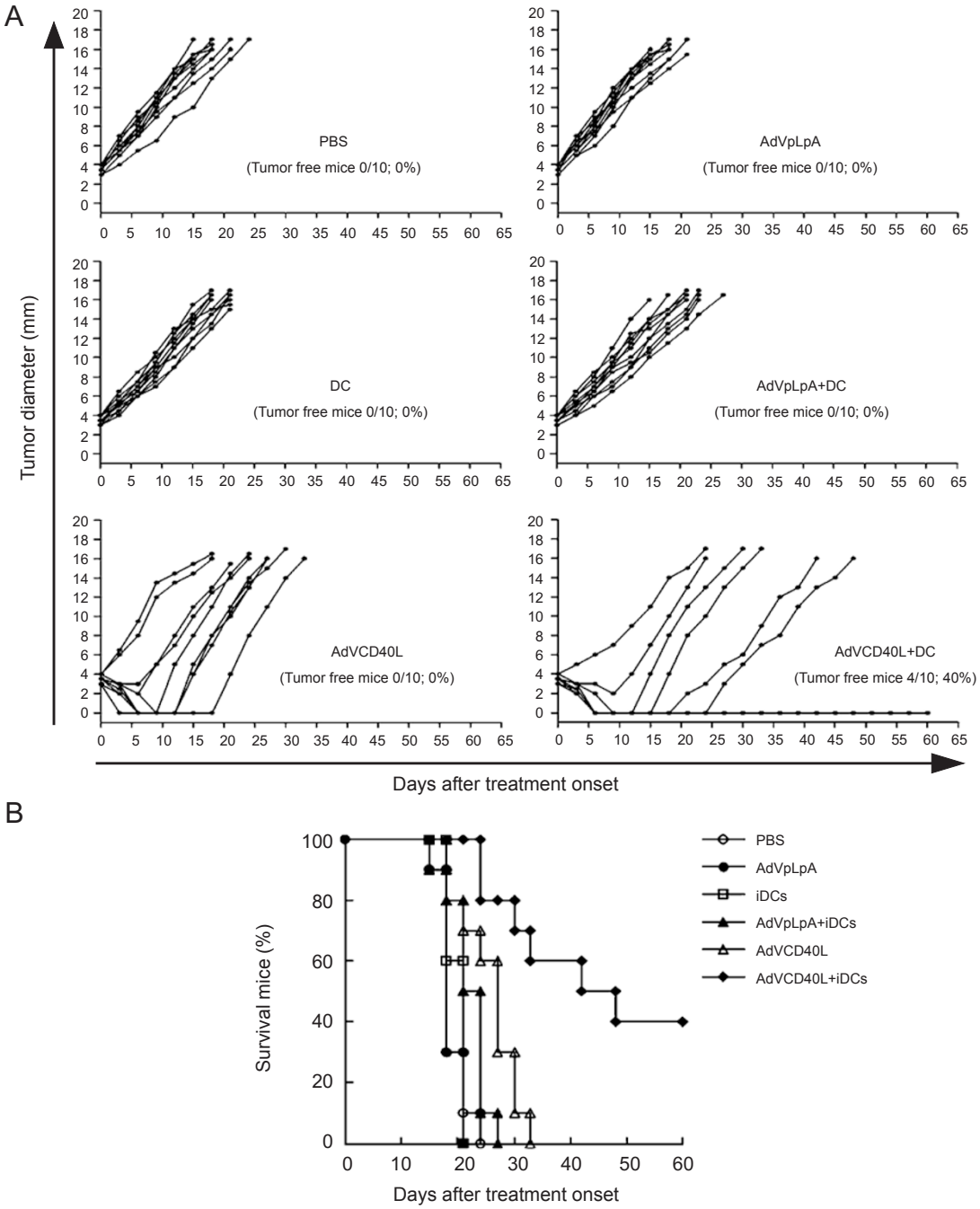

Figure 4 Inhibition of tumor growth (in mice with established tumors) by treatment with AdVCD40L and iDCs vaccine. Mice bearing well-established J558 tumors (3 mm - 4 mm in diameter) were first given intratumoral injection of AdVCD40L or AdVpLpA (5×10^9 pfu), followed 2 days later by i.t. injection with iDCs. Another four control groups of mice were given, respectively, intratumoral injection of PBS, iDCs, control AdVpLpA, or AdVCD40L alone. **(A)** Tumor growth was monitored and the tumor size (diameter) measured using a caliper. The evolution of the tumors in individual mice is depicted, as are the fractions of mice in each treatment group that were tumor free at 60 days post-treatment. **(B)** Tumor-bearing mice were observed for the duration of their survival period.

J558 tumor growth and induce tumor cell apoptosis *in vivo*. Based on this hypothesis, we set up experiments whereby iDCs were injected into tumor tissues to phagocytose the apoptotic cells or apoptotic bodies. CD40L expression in

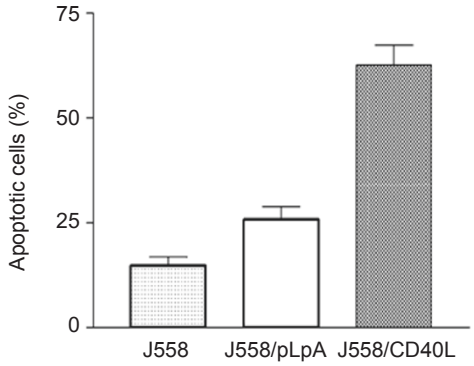

Figure 5 CD40L-induced apoptosis in J558 cell line. After transfection with AdVCD40L, apoptosis of J558 cells was measured by cell staining with FITC-Annexin-V antibody and propidium iodide according to the manufacturer's instruction. Data represent the mean ± S.D. of three independent experiments.

the tumor microenvironment thus stimulated the iDCs that had engulfed the apoptotic bodies to migrate to the lymph nodes for maturation. The mDCs then effectively primed and activated the T lymphocytes.

More recently, to induce antitumor immunity in an established myeloma model [146], we investigated the strategy using i.t. injection iDCs following AdVCD40L vaccination. Our results demonstrated that, 2 days following AdVCD40L injection, i.t. treatment with iDCs not only significantly suppressed tumor growth but also eradiated the established tumors in 40% of the mice (Figure 4). The potent antitumor effect produced by the combination therapy correlated with high expression of MHC, costimulatory and Fas molecules on J558 cells, which was derived from CD40L transgene expression. In addition, transgene CD40L expression could dramatically induce J558 cell apoptosis. The study of the related mechanism demonstrated that the apoptotic rate of J558/CD40L cells was significantly increased after transduction with the AdVCD40L compared with untransfected J558 or J558/pLpA cells (62.5% vs 14.9%, 25.7%) (Figure 5). iDCs that effectively captured apoptotic bodies in vivo could induce DC maturation. Since the J558/CD40L cells contained a population of apoptotic cells, the uptake of apoptotic bodies derived from tumor cells by iDCs was evaluated. The enhanced ability of iDCs

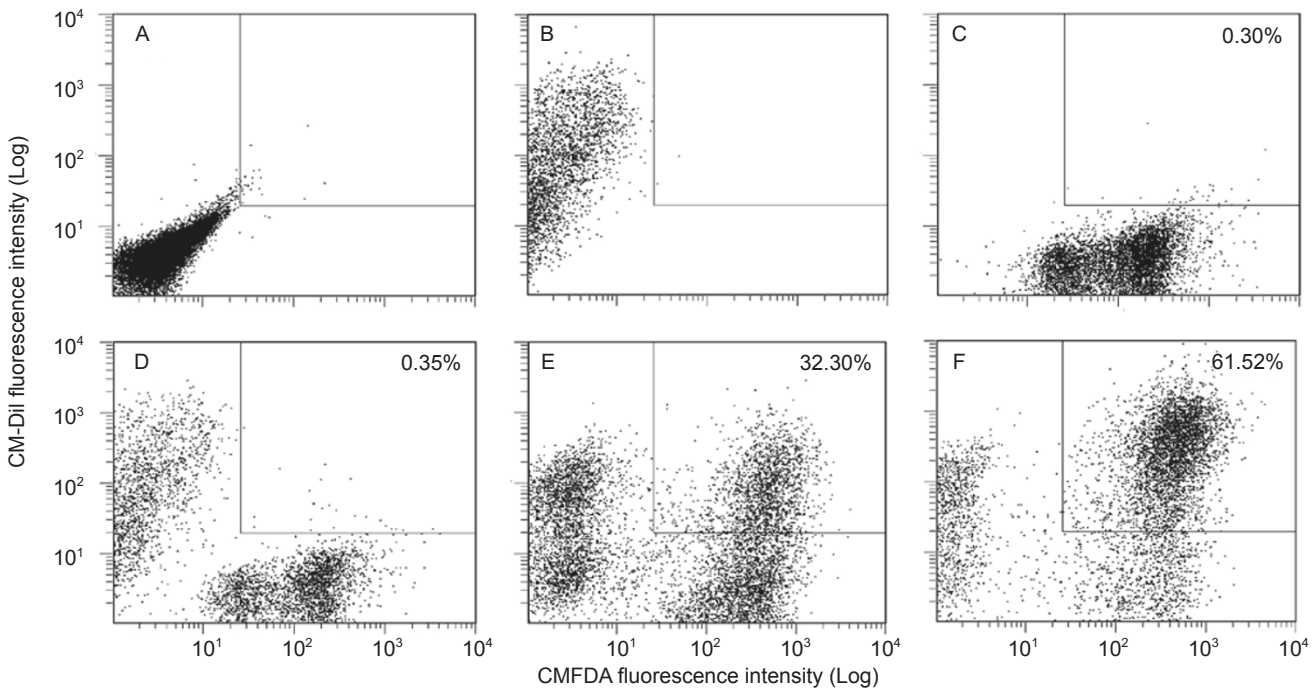

Figure 6 Uptake of apoptotic bodies derived from J558/CD40L by DCs. iDCs labeled with the CMFDA green fluorescent cell linker compound were co-cultured with J558/CD40L at a 1:1 ratio. They were recovered after transfection with AdVCD40L for 24 h, and then labeled with CM-DiI red fluorescent cell linker compound. The cells were analyzed by flow cytometry. **A**, negative (none-dying) DCs; **B**, J558/CD40L dying with CM-DiI; **C**, iDCs dying with CMFDA; **D**, **E** and **F** illustrate the uptake of apoptotic bodies by DCs at 37 ºC for 0h, 4h and 24h, respectively. The number shows the percent of dual color DCs, which have engulfed the apoptotic bodies of J558/CD40L cells.

to capture apoptotic bodies was observed in J558/CD40L cells and/or iDCs stained with red fluorescent CM-DiI and/or green fluorescent CMFDA. As shown in Figure 6, the percent of double positive cells was 32.3% and 61.5% after interaction with J558/CD40L for 4 h or 24 h, respectively. The effective capture of apoptotic bodies by iDCs *in vivo* could induce DC maturation, which in turn primed Th1 and tumor-specific CTL immune responses. To further elucidate the immune mechanism involved in the therapeutic immunity of AdVCD40L and iDCs vaccines, activated T cells were prepared as follows: splenocytes from mice were i.t. co-injected with AdVsCD40L and iDCs for two weeks, and then stimulated with irradiated J558 tumor cells for 4 days. These T cells were then subjected to phenotypic characterization by flow cytometry. The results showed that the activated T cells from mice treated with AdVCD40L and iDCs displayed a higher expression of T cell active marker (CD25) and costimulatory molecules CD40L when compared to T cells from the control mice. Interestingly, the FasL molecule was also significantly up-regulated. In summary, our data demonstrated that AdV-mediated CD40L gene therapy induced $CD40^+$ myeloma cells apoptosis and favored the maturation of iDCs through capturing the apoptotic cells, thus efficiently increasing the killing activity of tumor specific T lymphocytes. Therefore, it would appear that the sequential administration of AdVCD40L and iDCs may become a better paradigm for antitumor therapy.

Summary

Studies of the molecular pathogenesis and immunology of tumors have paved the way for the entrance of cancer gene therapy and immunotherapy into the mainstream of cancer treatment. Although the results of gene therapy or DC vaccines have been quite intriguing and provocative to date, the therapeutic efficiencies of these modalities are still far from satisfactory in well-established tumor animal models or most patients in clinical practice. Our findings and those of other investigators highlight the advantages of combined immunotherapy including AdV-mediated TNF-α or CD40L-gene therapy and DC vaccines in combating well-established tumors in animal models. This novel combined immunotherapeutic strategy may thus become a tool of considerable conceptual interest in the implementation of future clinical objectives. However, the potential benefits of administering cytokines or other DC activators in combination with DC vaccination remain to be further investigated.

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