

# Generation of Kidney Cancer-Specific Antitumor Immune Responses Using Peripheral Blood Monocytes Transduced With a Recombinant Adenovirus Encoding Carbonic Anhydrase 9

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## ABSTRACT

**Purpose:** Carbonic anhydrase 9 (CA9) is the most promising molecular marker described for renal cell carcinoma (RCC) to date. We investigated whether transduction of monocytes from peripheral blood with adenovirus encoding the CA9 gene (AdV-CA9) could stimulate a T-cell mediated immune response against cancer cells expressing CA9. The ability to consistently generate a T-cell response is an important step toward the development of a CA9-specific RCC vaccine.

**Experimental Design:** AdV-CA9 was generated using the AdEasy system. AdV-CA9-transduced peripheral blood mononuclear cell (PBMC)-derived monocytes were used to raise CTLs from autologous peripheral blood lymphocytes (PBLs). The ability of CTLs to lyse targets expressing CA9 was assessed by <sup>51</sup>Cr-release.

**Results:** Monocytes were efficiently transduced with AdV-CA9. In five of six experiments, AdV-CA9-transduced monocytes were able to induce a population of CTLs from bulk PBLs. CTLs were capable of lysing autologous, but not allogeneic monocytes expressing CA9. Furthermore, CTLs were able to lyse autologous RCC tumor cells expressing CA9. The ability of CTLs to lyse relevant targets was blocked by anti-CD3, anti-CD8, and anti-MHC class I antibodies demonstrating a MHC class I restricted response.

**Conclusions:** These results suggest that PBMC-derived monocytes transduced with AdV-CA9 can generate RCC-specific MHC class I restricted CTLs capable of lysing CA9-expressing cancer cells. Transduction of PBMC-

derived monocytes with adenovirus provides a simple and effective alternative to the use of dendritic cells for the induction of antigen-specific CTL.

## INTRODUCTION

Metastatic renal cell carcinoma (RCC) is resistant to conventional therapies, such as chemotherapy and radiation therapy (1). However, immunotherapy has demonstrated modest success in the treatment of patients with metastatic RCC. Cytokines such as IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , and interleukin 2 (IL-2), as well as cellular-based therapies such as lymphokine activated killer cells, CTLs, and natural killer cells have been reported to play important roles in immune responses against metastatic RCC (2, 3).

There is a need for new strategies of target-specific immunotherapy for the treatment of RCC because the overall long-term response rates to nonspecific immunotherapy with IL-2 are <10%. Although tumor-associated antigens (TAA) have recently been identified in RCC, few of these are commonly shared and can be used for clinical applications (4–6). To our knowledge, there are no RCC-associated antigens currently being investigated as therapeutic targets in the clinic.

Carbonic anhydrase 9 (CA9), a member of the carbonic anhydrase family, is a TAA found to be expressed in RCC that was initially discovered in HeLa (cervical cancer) cells (7). Expression of CA9 is induced by hypoxia and dysfunction of the *von Hippel-Lindau* (VHL) tumor suppressor, a key gene product involved in RCC that is located on the short arm of chromosome 3 (8). By immunohistochemistry analysis, >80% of primary and metastatic RCCs express CA9, whereas little to no expression is detected in normal kidneys (9). Furthermore, CA9 encodes both HLA class I- and II-restricted epitopes recognized by CTLs and helper T cells, respectively (10, 11). Moreover, expression of CA9 appears to be a favorable prognostic factor in patients with metastatic RCC (12). Therefore, it provides a rationale to investigate the induction of anti-CA9 T-cell responses in the immunotherapy of RCC. Previous studies of inducing gene-specific immune responses focused on dendritic cells (DCs) transduced with adenovirus encoding a TAA (13, 14). Other investigators have reported the feasibility of stably transduced human peripheral monocyte derived DCs with adenoviral vectors carrying granulocyte macrophage colony-stimulating factor,  $\beta$ -galactosidase, human papillomavirus antigens, as well as several melanoma antigens (15–19). However, low yield from peripheral blood mononuclear cells (PBMCs), prolonged culture time, and inconsistent results are associated with approaches involving DCs and, therefore, have hampered their potential application in the clinic.

In this study, we investigated the ability of PBMC-derived

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monocytes transduced with recombinant adenovirus carrying the CA9 gene (AdV-CA9) to stimulate CTL response. Use of an adenoviral vector is convenient because of its broad host range, safety profile, and persistent transgene expression *in vivo*. The use of monocytes may represent a simplified approach over DC-based methods, which may have broad utility in the clinic.

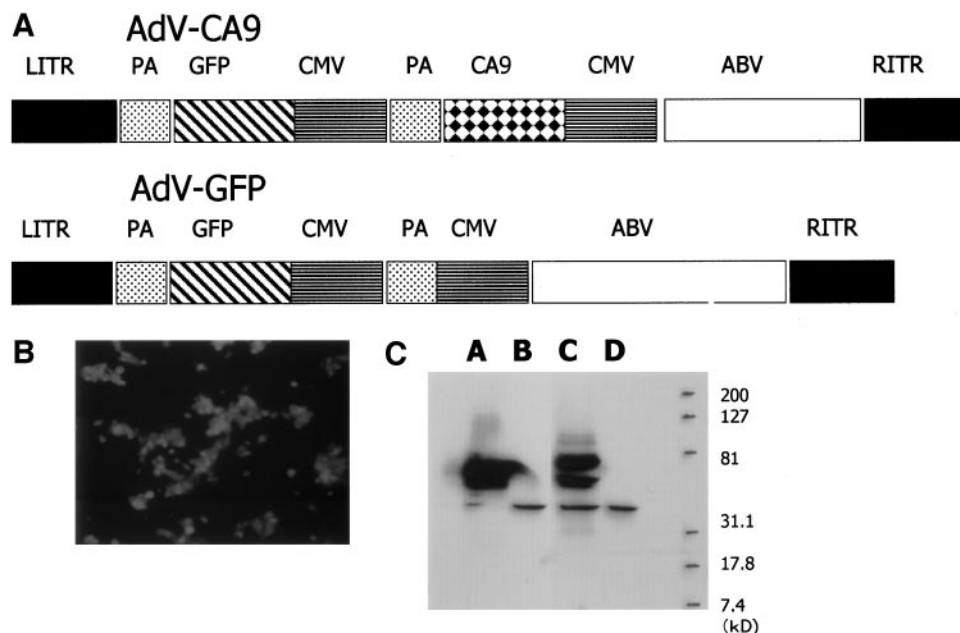
## MATERIALS AND METHODS

**Cloning of CA9 Gene Into a Recombinant Adenovirus Vector.** CA9 gene was amplified by PCR (Roche Expand High Fidelity PCR System) using pVL1393/granulocyte macrophage CA9 as a template (20). The specific primers used were: (5'-TCAGGTACCATGGCTCCCCTGTGCCCCAG-3', 5'-ATCCTCGAGCTAGGCTCCAGTCTCGGCTACCTC-3') containing *KpnI* and *XhoI* sites (underlined), respectively. The PCR product was subcloned into a pGEM-T-Easy vector (Promega, Madison, WI) to create pGEM-T-Easy-CA9. The plasmids pGEM-T-Easy-CA9 and pAdTrack-cytomegalovirus [CMV; AdEasy system; containing green fluorescent protein (GFP) and a CMV promoter] were digested using *KpnI* and *XhoI* and religated, resulting in pAdTrack-CMV-CA9. The AdEasy system was used for generation of the recombinant adenovirus (21). The resultant pAdTrack-CMV-CA9 encoded a CA9 gene under the control of a CMV promoter followed by a GFP gene under the control of a second CMV promoter. This plasmid pAdTrack-CMV-CA9 was cotransformed into electro-competent BJ5183 bacteria (22) with pAdEasy-1 (containing the viral backbone) and selected on Kanamycin LB plates. The plasmid in the bacteria was amplified and purified using a plasmid maxiprep system (Qiagen, Valencia, CA). The complete adenovector was linearized and used for transfection of 293 cells (human embryonic kidney cell line), where viral particles were further amplified, purified, and titered according to GFP-positive units. Southern blot analysis was used to confirm the presence of the CA9 gene. The resultant recombinant adenovirus,

AdV-CA9, was digested with *KpnI* and *XhoI* and electrophoresed on a 1.5% agarose gel and transferred to a filter and hybridized using a digoxigenine-labeled CA9 probe (Boehringer Mannheim GmbH, Germany).

**Generation and Transduction of Monocytes and Preparation of Responder Cells.** Autologous PBMCs from whole blood of healthy donors (kindly supplied by Dr. Gayle Baldwin, University of California Los Angeles) or kidney cancer patients were collected, followed by centrifugation over a Ficoll-Hypaque gradient (Pharmacia, Sweden). On day 0, cells were washed and resuspended in complete media (CM) containing RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% human serum (male, heat-inactivated; Omega Scientific, Tazana, CA), 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin at  $2 \times 10^6$ /ml and plated onto 100 mm  $\times$  20 mm tissue culture dishes (Corning Inc., Corning, NY) coated with human serum and incubated for 2 h at 37°C in a 5% CO<sub>2</sub> humidified incubator. Nonadherent cells were aspirated and replated in tissue culture dishes with CM for future use as responder cells. Adherent cells were further cultured overnight with CM at 37°C in a 5% CO<sub>2</sub> humidified incubator. Resultant nonadherent cells were harvested and analyzed by flow cytometry to confirm the presence of monocytes. On day 1, the nonadherent monocytes generated were resuspended in CM in tissue culture dishes and transduced overnight with AdV-CA9 at 1000 plaque-forming units/cell in CM at 37°C in a 5% CO<sub>2</sub> humidified incubator. Confirmation of AdV-CA9 transduction was based on GFP expression demonstrated by UV light microscopy and CA9 cell-surface expression demonstrated by flow cytometry.

**Flow Cytometry Analysis.** Monoclonal antibodies against the human molecules HLA-A2 (BB7.2; Becton-Dickinson, Mountain View, CA), HLA-DR, CD3, CD4, CD8, CD14 (all from Becton-Dickinson, San Jose, CA), CD56, CD83,



**Fig. 1** A, schematic representation of the cassettes expressing carbonic anhydrase 9 (CA9) and green fluorescent protein (GFP) in constructs of adenoviruses encoding the CA9 and GFP genes (AdV-CA9 and AdV-GFP, respectively). Both CA9 and the downstream GFP gene were under the control of two different cytomegalovirus promoters. B, detection of CA9 expression on 293 cells using green fluorescent microscopy. C, detection of CA9 expression using Western blot analysis. Lane A, R6, a CA9-expressing human kidney cancer cell line; Lane B, R11, a non CA9-expressing human kidney cancer cell line; Lane C, AdV-CA9-transduced 293 cells; and Lane D, nontransduced 293 cells. LITR, left inverted terminal repeat; RITR, right inverted terminal repeat; PA, polyadenylation site; CMV, cytomegalovirus promoter.

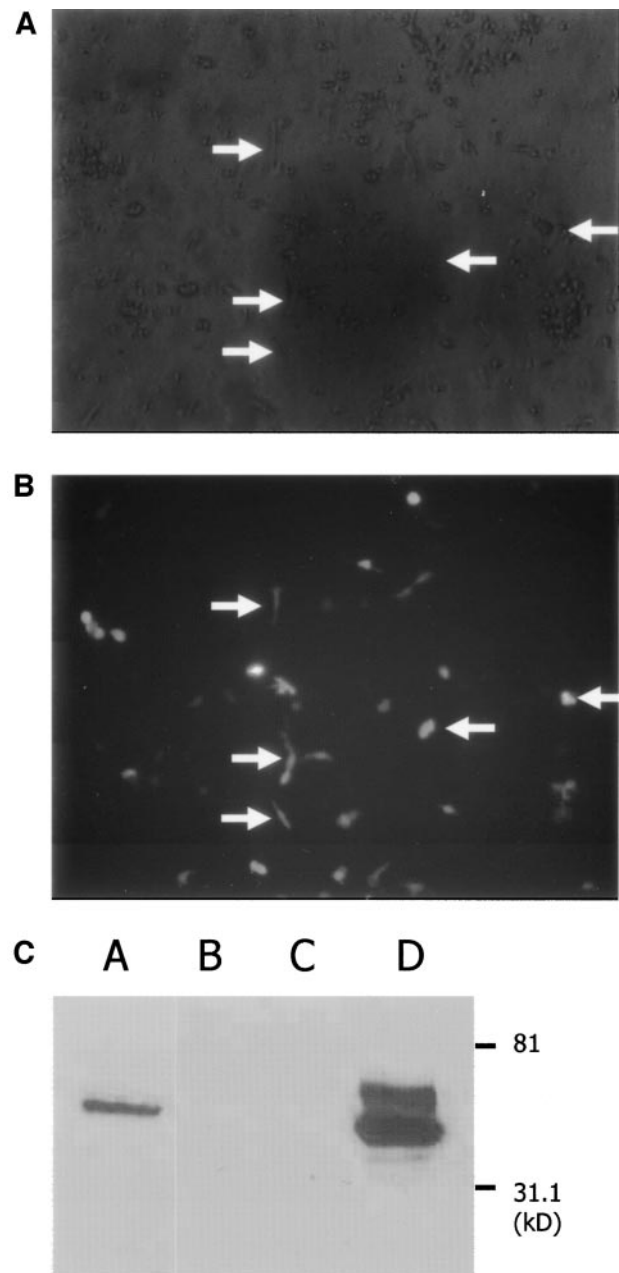
CD86, TcR $\alpha\beta$ , CA9 (M75; from Dr. Eric Stanbridge, University of California Irvine), and appropriate isotype controls (PharMingen, San Diego, CA) were obtained directly labeled with phycoerythrin or FITC. For phenotypic analysis,  $1 \times 10^6$  stimulator, responder, and target cells were double stained with the indicated antibodies. Propidium iodide was used to exclude dead cells from the analysis. Five to 10,000 events/sample were acquired on a Becton-Dickinson FACScan II flow cytometer that simultaneously acquires forward and side scatter, as well as FL1 (FITC) and FL2 (phycoerythrin) data, and analyzed using the CellQuest Software (Becton-Dickinson, San Jose, CA). All parameter settings were optimized at the initiation of the study and were maintained constant throughout subsequent analyses.

**Western Blot Analysis.** CA9 protein expression was determined by Western blot analysis. Briefly, cells were harvested after incubation with 0.05% trypsin and 0.53 mM EDTA, washed twice with cold PBS, resuspended in lysis buffer [50 mM Tris-HCl (pH7.4), 0.1 mM EDTA, 0.5% Triton X-100, 1 mM DTT, 10% of a protease inhibitor cocktail, Sigma], incubated for 15 min at room temperature and centrifuged at  $12,000 \times g$  for 10 min. Supernatants underwent electrophoresis on a 12% SDS-polyacrilamide gel. Proteins were transferred to a nitrocellulose membrane by electrophoresis and probed with the primary antibody (anti-CA9 antibody, M75, 1:5000 dilution, provided by Dr. Eric Stanbridge) and HRP-conjugated secondary antibody (1:2000 dilution). Nitrocellulose membranes were developed using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, United Kingdom) and exposed to film (Kodak).

**Generation of RCC Cell Lines.** Primary cultures from kidney cancer surgical specimens were established by a single-cell isolation method. Tumor specimens were minced into small pieces using sterile scalpels. Tumor digestion was performed using 100 mg/ml of collagenase, 10 mg/ml of hyaluronidase, and 10 mg/ml of DNase overnight in tissue culture dishes. Tumor cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics for 7 days and stimulated with IFN- $\gamma$  (50 units/ml) for an additional 2 days before use.

**In Vitro T-Cell Stimulation.** For CTL generation, a single stimulation was performed by coculturing  $1 \times 10^4$  AdV-CA9-transduced monocytes, nontransduced monocytes, or no stimulators with  $3 \times 10^3$ ,  $1 \times 10^4$ , and  $3 \times 10^4$  PBLs, respectively, in 200  $\mu$ l of media in each well for 7 days in V-bottomed microculture plates in groups of 24 for each stimulation condition at 37°C. Media contained RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum, HEPES buffer (10 mM), 2-mercaptoethanol ( $5 \times 10^{-5}$  M), antibiotics, recombinant human IL-2 (5 units/ml) and recombinant human IFN- $\gamma$  (50 units/ml). Monocytes were treated with mitomycin C (50  $\mu$ g/ml for 30 min) before use. After 7 days of coculturing, lymphocytes from each group were harvested, mixed, and tested for the ability to lyse various target cells.

**Cytotoxicity Assays.** Target cells (adenovirus-transduced monocytes or tumor cells) stimulated with IFN- $\gamma$  (50 units/ml) for 2 days were harvested and labeled with 200  $\mu$ Ci of Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> for 90 min and washed three times before use. Lymphocytes stimulated with transduced monocytes in microculture wells were harvested and mixed in graduated doses with  $3 \times 10^3$  labeled target cells in 150  $\mu$ l media (RPMI 1640



**Fig. 2** A, phase-contrast microscopy demonstrating large nonadherent monocyte cells (arrows pointing left) and spindle-shaped adherent monocyte cells (arrows pointing right). B, fluorescent microscopy showing green fluorescent protein expression from monocytes transduced with adenovirus encoding both the CA9 and GFP genes (AdV-CA9). C, Western blot analysis demonstrating expression of carbonic anhydrase 9 (CA9) protein in AdV-CA9-transduced monocytes. Lane A, R6, a CA9-expressing human kidney cancer cell line; Lane B, R11, a non-CA9 expressing human kidney cancer cell line; Lane C, nontransduced monocytes; and Lane D, AdV-CA9-transduced monocytes.

supplemented with 5% fetal bovine serum) in round-bottomed 96-well tissue culture plates. Cells were incubated for 4 h at 37°C in a 5% CO<sub>2</sub> humidified incubator, and the <sup>51</sup>Cr released from target cells was measured with a gamma counter and was

Table 1 Cell-surface phenotype of peripheral blood lymphocytes and monocytes from individual donors<sup>a</sup>

Donor	Peripheral blood lymphocytes					Monocytes (%)			
	CD3	CD4	CD8	CD56	T-cell receptor $\alpha\beta$	HLA-DR	CD83	CD86	HLA-A2
1	70.8	39.1	24.2	8.9	69.2	ND	42.6	69.8	ND <sup>b</sup>
2	74.4	57.4	13.8	14.7	73.9	ND	17.1	24.0	P
3	63.3	31.9	17.8	16.2	58.7	83.8	22.2	79.9	N
4	63.9	43.6	23.4	17.1	60.6	93.5	8.5	87.3	P
5	73.8	38.6	27.8	15.2	68.5	73.2	41.1	64.1	P

<sup>a</sup> Cells were double stained with the indicated antibodies and analyzed by flow cytometry with isotype controls.

<sup>b</sup> ND, not determined; P, positive; N, negative.

defined as the observed counts minus spontaneous counts divided by the total releasable counts minus spontaneous counts.

Cytotoxicity assays were also performed in the presence of blocking antibodies for PBLs stimulated with AdV-CA9-transduced monocytes. Monoclonal antibodies used in the blocking studies were antihuman CD3 (HIT3a, BD Biosciences, CA), antihuman CD4 (S3.5), antihuman CD8 (3B5), antihuman HLA class I (Tu149), and antihuman HLA class II (TU36; all purchased from Caltag, Burlingame, CA). The antibodies were either mouse IgG2a or IgG2b. Significant differences between the cytolytic activities of each experimental group were estimated by the Mann-Whitney *U* test (23).

**Generation of T-cell Clones and Cytokine-Release Assays.** T-cell clones were generated by limiting dilution (20 cells/well) from healthy donor PBL-derived by using AdV-CA9-transduced autologous monocytes ( $1 \times 10^3$  cells/well) as stimulator cells in 200  $\mu$ l of CM supplemented with 5 IU/ml IL-2 and 50 IU/ml IFN- $\gamma$  in U-bottomed 96-well tissue culture plates. T-cell clones were restimulated weekly for 2 weeks with AdV-CA9-transduced autologous monocytes ( $1 \times 10^3$  cells/well) by replating into new U-bottomed 96-well tissue culture plates. Fresh media with 5 IU/ml IL-2 and 50 IU/ml IFN- $\gamma$  were added twice a week to each well of cells. One week after the last restimulation, lymphocytes were harvested and mixed with adenovirus-transduced autologous PBMCs. Cellular immune responses were monitored with cytotoxicity and IFN- $\gamma$  release assays.

For IFN- $\gamma$  release assays, T-cell clones ( $3 \times 10^4$ ) and

adenovirus-transduced autologous PBMCs ( $3 \times 10^4$ ) were mixed in 200  $\mu$ l CM in V-bottomed microculture plates at 37°C in a 5% CO<sub>2</sub> humidified incubator. After 24 h, the supernatant was collected and analyzed for IFN- $\gamma$  with an ELISA kit (Endogen, Woburn, MA). Micro-well cultures were considered to be positive if they secreted >100 pg/ml of IFN- $\gamma$  against AdV-CA9-transduced PBMCs and at least 2-fold the amount of IFN- $\gamma$  against AdV-GFP-transduced PBMCs (negative control). IFN- $\gamma$  secretion was corrected for by subtracting the background of IFN- $\gamma$  secreted in transduced target cells.

## RESULTS

### Generation of AdV-CA9 and Transduction of 293 Cells.

The AdEasy system was used to generate AdV-CA9 as described in "Materials and Methods." The adenovirus construct consisted of two separate CMV promoters driving the expression of CA9 and GFP proteins (Fig. 1A). Evidence for successful gene cloning of AdV-CA9 was demonstrated by Southern blot analysis (data not shown). The 293 cells were infected at a multiplicity of infection of 1000. Transduction efficiency AdV-CA9 for 293 cells was  $\geq 99\%$  by green fluorescent microscopy (Fig. 1B) and flow cytometry (data not shown). Western blot analysis was performed to confirm CA9 protein expression in 293 cells after transduction with AdV-CA9 (Fig. 1C). Lane C demonstrates the 54-kDa band corresponding to the CA9 protein.

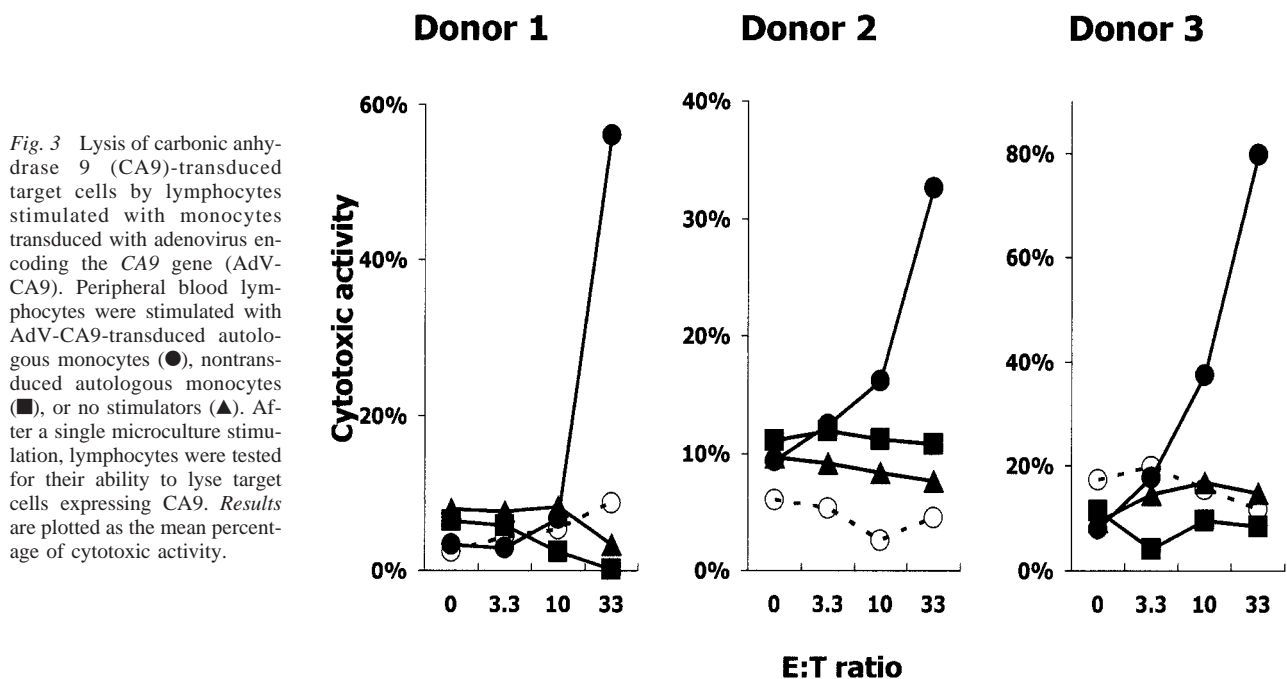
### Expression of CA9 Protein in Human Monocytes Transduced with AdV-CA9.

Monocytes derived from autologous PBMCs of healthy donors were used as antigen presenting cells. Approximately  $5\text{--}10 \times 10^6$  monocytes were obtained from  $1 \times 10^8$  fresh PBMCs. Monocytes demonstrated characteristic morphology (Fig. 2A) and expression of cell surface markers with high expression of MHC class II molecules ranging from 73% to 94%, high expression of costimulatory molecules ranging from 9% to 43% for CD83 and 24% to 87% for CD86 (Table 1). Successful adenoviral transduction was confirmed by analyzing GFP expression using fluorescent light microscopy. Monocytes expressed GFP, which was downstream of CA9, indicating efficient transduction (Fig. 2). Western blot analysis confirmed CA9 protein expression of AdV-CA9-transduced monocytes and R6, which is a RCC tumor cell line expressing CA9 (Fig. 2C). Under the same conditions, CA9 expression was not present in R11, a RCC tumor cell line that does not express CA9. AdV-CA9 transduction efficiency of monocytes used as target cells varied among samples from

Table 2 Transduction of monocytes and target cells from individual donors<sup>a</sup>

Donor	Monocytes (%)		Monocytes used as target cells (%)	
	AdV-CA9 ( <i>GFP</i> )	AdV-CA9 ( <i>CA9</i> )	AdV-CA9 ( <i>CA9</i> )	AdV-GFP ( <i>GFP</i> )
1	31.0	38.1	38.1	35.8
2	41.7	26.6	26.6	24.8
3	57.5	68.3	68.3	48.3
4	46.0	93.1	93.1	67.9
5	48.7	22.6	22.6	24.2

<sup>a</sup> Monocytes were transduced with AdV-CA9, which encodes both the CA9 and the GFP gene under the control of two separate CMV promoters. Monocytes were examined under fluorescent light microscopy for expression of GFP. Target cells were transduced with AdV-CA9 or AdV-GFP. Target cells were stained with labeled anti-CA9 antibody (M75) against CA9, and cell-surface expression was determined by flow cytometric analysis with isotype controls or cells were examined under fluorescent light microscopy for GFP expression.



**Fig. 3** Lysis of carbonic anhydrase 9 (CA9)-transduced target cells by lymphocytes stimulated with monocytes transduced with adenovirus encoding the CA9 gene (AdV-CA9). Peripheral blood lymphocytes were stimulated with AdV-CA9-transduced autologous monocytes (●), nontransduced autologous monocytes (■), or no stimulators (▲). After a single microculture stimulation, lymphocytes were tested for their ability to lyse target cells expressing CA9. Results are plotted as the mean percentage of cytotoxic activity.

different donors, ranging from 22% to 93% of the whole monocyte population (Table 2).

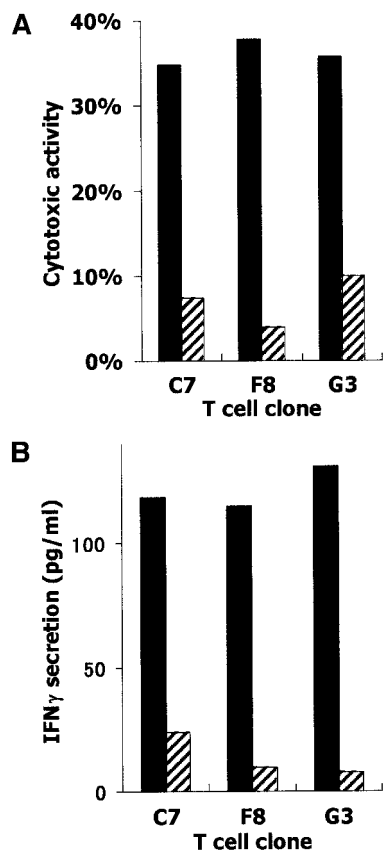
**AdV-CA9-Transduced Monocytes Induce Cell-Mediated Cytotoxicity.** To generate CTLs, AdV-CA9-transduced monocytes were used to stimulate autologous PBLs from individual healthy donors using the microculture technique described in "Materials and Methods." One week after coculturing, cytolytic activity was measured with cytotoxicity assays. PBLs stimulated with either autologous AdV-CA9-transduced monocytes, autologous nontransduced monocytes, or no stimulation were tested for cytolytic activity against autologous monocyte targets expressing CA9. PBLs stimulated with AdV-CA9-transduced monocytes (Fig. 3, ●) were able to lyse autologous monocyte target cells expressing CA9 and were unable to lyse autologous target cells expressing GFP (Fig. 3, ○). In addition, PBLs stimulated with nontransduced monocytes (Fig. 3, ■) or without stimulation (Fig. 3, ▲) did not show a cytolytic response against autologous target cells expressing CA9. These results suggest that AdV-CA9-transduced monocytes induce CTLs capable specifically lysing cells expressing CA9.

To further demonstrate the specificity against CA9, T-cell clones were generated by limiting dilution from lymphocytes derived from bulk PBLs of one healthy donor stimulated with AdV-CA9-transduced autologous monocytes as described above. T-cell clones (20 cells/well) were restimulated weekly for 2 weeks by adding AdV-CA9-transduced autologous PBMCs as feeders. At the end of the third week, the resultant T-cell clones (or clods) were tested for their ability to lyse relevant targets and induce antigen-specific cytokine release. Three of 96 clones (or clods) demonstrated specific lytic activity against target cells expressing CA9, but not against control cells expressing GFP (Fig. 4A). In addition, they were able to induce CA9-specific cytokine release (Fig. 4B).

**Cytotoxicity Against CA9 Is MHC Restricted.** The cytolytic response was blocked using different blocking antibodies against molecules involved in the immune response to provide evidence that cytolysis was MHC restricted. CTLs were individually generated from the PBLs of four healthy donors. PBLs were stimulated with AdV-CA9-transduced autologous monocytes using the coculture technique described above. In the presence of anti-CD3 antibody (Fig. 5, ◆), anti-CD8 antibody (Fig. 5, ■), or anti-MHC class I antibody (Fig. 5, ▲), cytolytic activity against monocyte target cells expressing CA9 was markedly reduced. In contrast, addition of anti-CD4 (Fig. 5, □) or anti-MHC class II (Fig. 5, △) antibodies had little or no inhibition of cytolysis. In summary, the blocking experiments provided evidence that recognition of CA9 was in the context of MHC class I molecules.

CTLs generated from AdV-CA9-transduced autologous monocytes were also tested against allogeneic target cells expressing CA9. PBLs for individual healthy donors were stimulated with AdV-CA9-transduced autologous monocytes to yield a population of CTLs. CTLs generated were able to significantly lyse autologous, but not allogeneic target cells expressing CA9 (Fig. 6). Therefore, CTLs generated from AdV-CA9-transduced monocytes appeared to recognize antigen in the context of self-MHC. A modest cytolytic effect against allogeneic target cells (donor 4) was observed with CTLs generated from donor 5 (Fig. 6, ■), but this might have been attributable to donor 4 and 5 both sharing the same MHC class I haplotype HLA-A2 (Table 1).

**CTLs Can Lyse RCC Tumor Expressing CA9.** To demonstrate that the monocyte stimulation approach could generate CTLs against RCC tumor, *in vitro* T-cell stimulation was carried out in three RCC patients (patients B, C, and D) and one transitional cell carcinoma patient (patient A). PBLs obtained



**Fig. 4** Lysis of carbonic anhydrase 9 (CA9)-transduced target cells and CA9-specific cytokine release by T-cell clones generated *in vitro* by restimulation with autologous monocytes transduced with adenovirus encoding the CA9 gene (AdV-CA9). CA9-specific T-cell clones were generated by limiting dilution from healthy donor peripheral blood lymphocytes stimulated with AdV-CA9-transduced autologous monocytes. After two weekly restimulations, the lymphocytes were tested for their ability to recognize relevant targets. Target cells were autologous peripheral blood mononuclear cells transduced with AdV-CA9 (■) or AdV-green fluorescent protein (▨). Three of the 96 wells tested showed specific activity against target cells expressing CA9. **A**,  $\sim 1 \times 10^5$  T-cell clones (or clods) were harvested and mixed with  $3 \times 10^3$   $^{51}\text{Cr}$ -labeled target cells. Cells were incubated for 4 h at 37°C in a 5%  $\text{CO}_2$  incubator.  $^{51}\text{Cr}$  release from the lysed target cells was measured. **Results** are plotted as the mean percentage of cytotoxic activity. **B**,  $3 \times 10^4$  T cell clones (or clods) were cocultured  $3 \times 10^4$  target cells transduced with AdV-CA9 or adenovirus encoding the GFP gene (AdV-GFP). After 24 h, the supernatant was collected and analyzed for human IFN- $\gamma$  secretion. IFN- $\gamma$  secretion was corrected for by subtracting the background of IFN- $\gamma$  secreted in transduced target cells.

from patients were stimulated with autologous AdV-CA9-transduced monocytes or autologous nontransduced monocytes supplemented with IL-2 and IFN- $\gamma$  for 7 days. AdV-CA9-transduced monocytes were able to generate CTL against autologous tumor cells expressing CA9 (patients B and D; Fig. 7A), but not against autologous tumor cells lacking CA9 expression (patients A and C; Fig. 7A). Transitional cell carcinoma (patient A) and RCC (patients B, C, D) tumors were characterized for CA9 expression with Western blot analysis. RCC tumor from patients B and D demonstrated CA9 expression, whereas tumors from patients A and C did not (Fig. 7B).

## DISCUSSION

RCC and melanoma are two major types of cancer that respond to immunotherapy. In melanoma, identification of TAAs in the last decade has opened new opportunities to develop improved immunotherapy approaches (24). However, the lack of shared TAAs in RCC and practical approaches to induce CTLs from candidate TAAs hamper development of targeted immunotherapy for RCC. Recently, recombinant adenoviruses encoding TAAs have been successfully used to induce CTLs against candidate melanoma antigens by a number of investigators (14, 18, 25). These studies have mainly focused on the use of human DCs as antigen-presenting cells. However, generation of DCs requires a complicated, lengthy cell culture and often results in low yields from patient PBMCs. In addition, trivial changes in culturing conditions, such as use of different serum or presence of residual cytokines can give rise to markedly different populations of DCs and thus inconsistent results for T-cell generation.

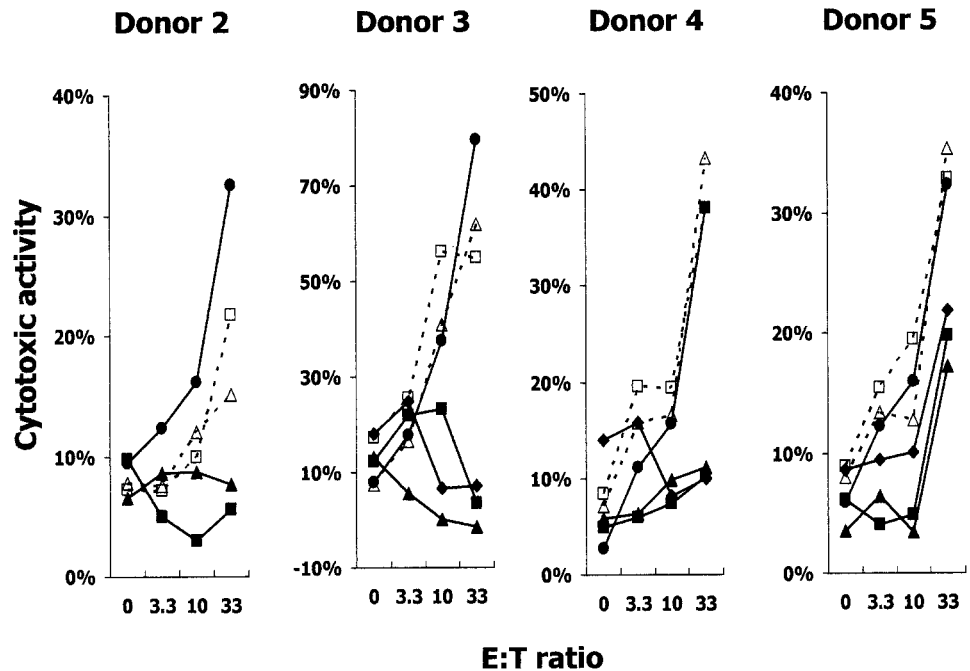
In this study, we used PBMC-derived monocytes as antigen-presenting cells for adenovirus transduction and CTL generation. Monocytes were generated from PBMCs using a simple cell-attachment procedure without the requirement of cytokine induction or lengthy cell culturing. Monocytes generated *in vitro* demonstrated high expression of MHC class II and costimulatory molecules and could be efficiently transduced by adenoviruses.

Using adenovirus-transduced monocytes, we were able to generate CA9-specific CTLs from PBLs in an autologous system. Previous reports showed that CA9 antigen could be restricted by HLA-A2 (9, 10), and possibly other MHC class I molecules in addition to HLA-A2. The approach shown in this study was able to generate CA9 specific CTLs from HLA-A2 donors as well as those that were not HLA-A2 and, therefore, may be useful as a general strategy for generating CTLs without previous knowledge of patient HLA types. In addition, CTLs generated from donor 5 were capable of lysing allogeneic target cells from donor 4. Similarly, CTLs generated from donor 4 were capable of lysing allogeneic target cells from donor 5, suggesting that epitopes from CA9 shared by both donors might exist. The induced CTLs were possibly polyclonal, and some might have been restricted by the shared *HLA-A2* allele. In addition to functioning as stimulators, it may be possible to use adenovirus-transduced monocytes as antigen-presenting cells for monitoring specific T-cell responses in patients with any HLA class I allele and without defined peptide epitopes from CA9.

It is possible that adenovirus-transduced monocytes may yield a population of nonspecific natural killer cells (26–28). IL-2 and IFN- $\gamma$  at high doses are both potentially able to raise natural killer cell activity (29, 30). Although IL-2 and IFN- $\gamma$  were added in culture medium in our experimental system, the activity of activated natural killer cells was probably minimal because only low concentrations of IL-2 and IFN- $\gamma$  were used in the cell culture.

Previous approaches to induce CA9-specific CTLs in our laboratory used DCs transduced with adenovirus encoding granulocyte macrophage colony-stimulating factor-CA9 and DCs cocultured with the granulocyte macrophage colony-stimulating

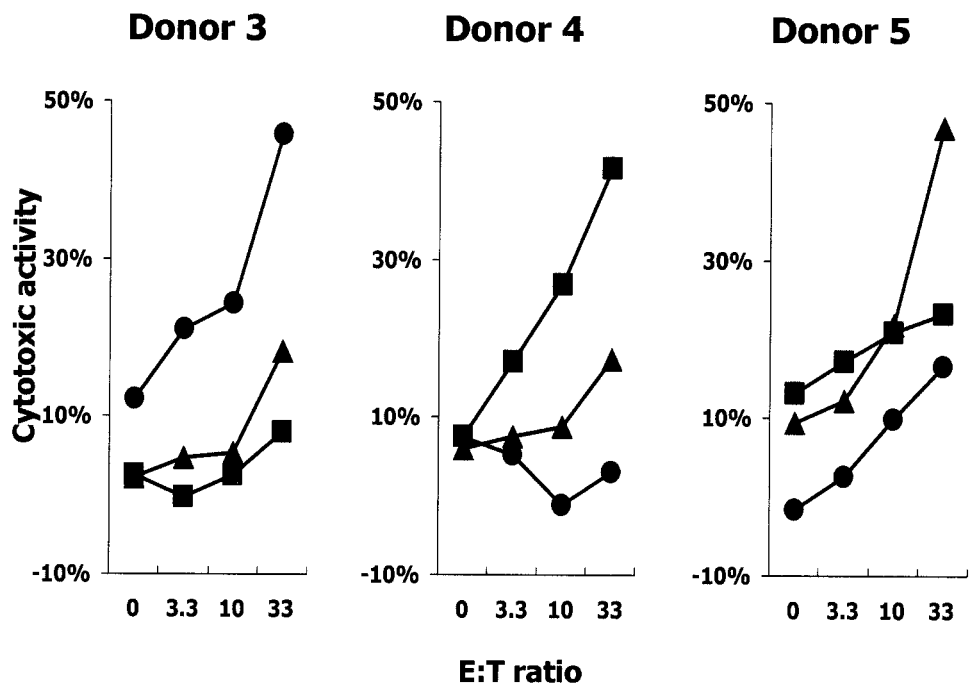
**Fig. 5** Lysis of carbonic anhydrase 9 (CA9)-transduced target cells in the presence of blocking antibodies by lymphocytes stimulated with monocytes transduced with adenovirus encoding the CA9 gene (AdV-CA9). PBLs were stimulated with AdV-CA9-transduced autologous monocytes. After a single microculture stimulation, lymphocytes were tested for their ability to lyse target cells expressing CA9 in the presence of: no antibodies (●); anti-HLA class I antibody (▲); anti-HLA class II antibody (△); anti-CD3 antibody (◆); and anti-CD4 antibody (□); and anti-CD8 antibody (■). Donors 2, 3, 4, and 5 were derived from donors 2, 3, 4, and 5 in Table 1, respectively. Results are plotted as the mean percentage of cytotoxic activity.

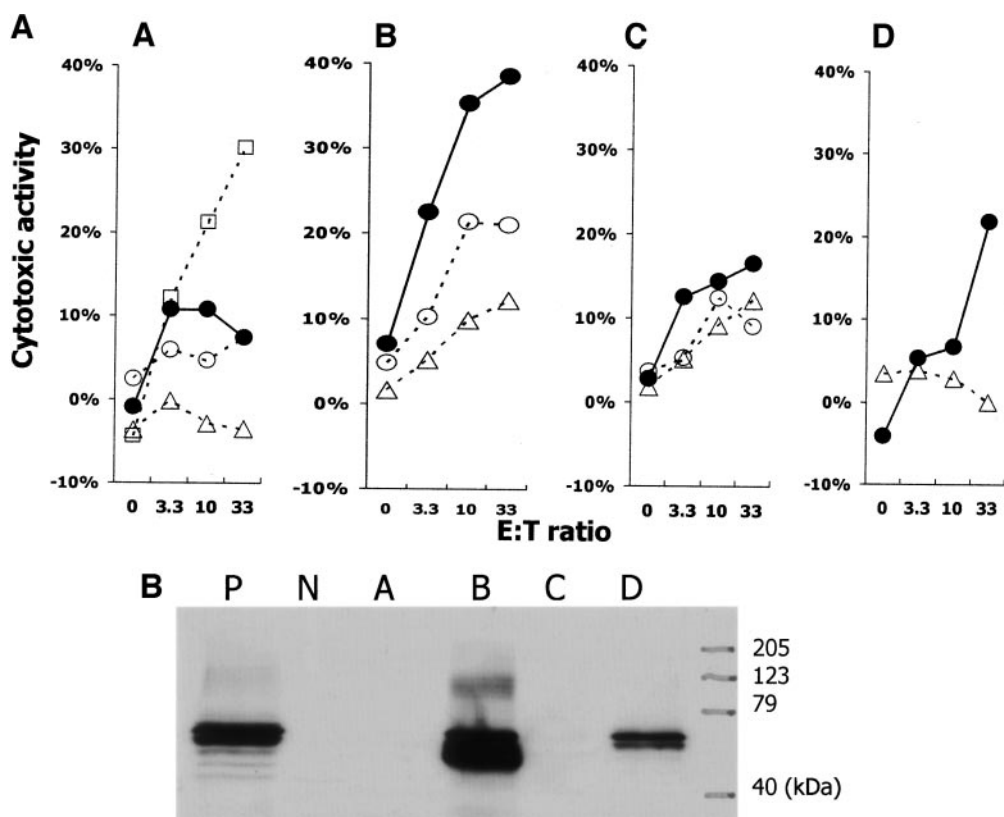


factor-CA9 fusion protein. All three approaches demonstrated evidence for generation of CA9-specific CTLs. Compared with our previous approaches, the current approach involves the least amount of cell manipulation and the shortest time for cell culture. Therefore, it may be a more practical approach for the following clinical applications: (a) adoptive cell therapy, autologous monocytes from metastatic RCC patients may be trans-

duced with AdV-CA9 *in vitro*, and induced CTLs may be isolated and adoptively transferred back into patients; and (b) active immunization, monocytes can be obtained from metastatic RCC patients, transduced with AdV-CA9 *in vitro*, and used as a vaccine for those patients. Although the use of this approach to generate specific CTLs as an alternative to DCs has been successfully performed thus far only *in vitro*, these results

**Fig. 6** Lysis of carbonic anhydrase 9 (CA9)-transduced target cells by lymphocytes stimulated with monocytes transduced with adenovirus encoding the CA9 gene (AdV-CA9) is MHC restricted. Peripheral blood lymphocytes were stimulated with AdV-CA9-transduced autologous monocytes and the CTLs were tested for ability to lyse monocyte target cells expressing CA9 derived from either donor 3 (●), donor 4 (■), or donor 5 (▲). Results are plotted as the mean percentage of cytotoxic activity.





**Fig. 7** A, lysis of autologous kidney cancer cells expressing carbonic anhydrase 9 (CA9) by lymphocytes stimulated with monocytes transduced with adenovirus encoding the CA9 gene (AdV-CA9). Peripheral blood lymphocytes were stimulated with AdV-CA9-transduced autologous monocytes (●), nontransduced autologous monocytes (○), or no stimulator cells (△). Target cells were derived from upper tract transitional cell carcinoma (TCC) or renal cell carcinoma (RCC) tumor specimens. Autologous PBMCs expressing CA9 were used as target cells for a positive control (□) in patient A. A–D represent patient A (upper tract TCC) and patients B, C, and D (RCC), respectively. Results are plotted as the mean percentage of cytotoxic activity. B, Western blot analysis demonstrating expression of CA9 protein in TCC and RCC tumor specimens. Lane P, R6, a CA9-positive human kidney cancer cell line; Lane N, R11, a CA9-negative human kidney cancer cell line; Lane A, upper tract TCC tumor derived from patient A; Lanes B–D, RCC tumors derived patients B, C, and D, respectively.

may have implications for use in human tumor immunotherapy. These approaches are currently being investigated in our laboratory in animal models. In addition, we acknowledge that the use of DCs has not been directly compared with this approach to test for differences between the two methods.

Overall, we demonstrated the feasibility of using adenovirus-transduced monocytes for the induction of lytic CTLs against CA9 in healthy donors and RCC patients. Adenovirus-transduced monocytes may represent an attractive alternative to DC-based approaches in the stimulation of CTLs from PBL, which may have broad application in tumor immunotherapy.

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