

# Novel Kidney Cancer Immunotherapy Based on the Granulocyte-Macrophage Colony-stimulating Factor and Carbonic Anhydrase IX Fusion Gene

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## ABSTRACT

**Purpose:** We investigated the ability of the fusion protein granulocyte-macrophage colony-stimulating factor and carbonic anhydrase IX (GMCA-9)<sup>1</sup> to induce an immune response *in vitro* and *in vivo* for the development of a GMCA-9-based kidney cancer vaccine.

**Experimental design:** Human dendritic cells (DCs) were transduced with a recombinant adenovirus containing the GMCA-9 gene and tested for their capacity to induce CA9-specific cytotoxic T lymphocytes *in vitro*. Tumor growth was studied in severe compromised immunodeficiency disease (SCID) mice s.c. injected with R11-GMCA-9, a human renal cell carcinoma cell line stably transfected with the GMCA-9 gene. Involvement of natural killer (NK) cells in the antitumor activity of GMCA-9 was determined in SCID mice treated with the NK-blocking agent anti-asialoGM-1.

**Results:** DC and R11 cells transduced with GMCA-9 produced a GMCA-9 protein that is targeted to the cell membrane and partially processed to granulocyte macrophage colony-stimulating factor- and CA9-like products. Furthermore, GMCA-9 was capable of inducing DC maturation, as well as CA9-specific cytotoxic lymphocytes *in vitro*. Tumor growth of R11 cells in SCID mice was significantly inhibited after transfection with the GMCA-9 fusion gene ( $P < 0.01$ ). In mice treated with anti-asialoGM-1, R11-GMCA-9 tumors grew significantly faster than those of control mice ( $P < 0.05$ ), suggesting an involvement of NK cells.

**Conclusions:** Our results suggest that the fusion protein GMCA-9 is capable of generating an immune response both *in vitro* and *in vivo*. Additional studies will confirm the utility of *ex vivo* GMCA-9-transduced DCs as a kidney cancer vaccine.

## INTRODUCTION

The relative immunogenicity of RCC<sup>2</sup> and existence of RCC-specific, MHC-restricted CTLs in RCC patients (1) appear to indicate that RCC expresses antigenic determinants that can generate tumor-specific immune responses. Reports of spontaneous regressions and the demonstration of tumor-reactive CTLs indicate the importance of the host's immune system in patients with RCC (2). A limited number of kidney cancer-specific tumor antigens has been identified thus far, including a mutated HLAA2 protein (3) and a renal tumor antigen protein (4). More recently, a renal antigen known as G250 (5) has been identified as the first widely expressed tumor antigen for RCC (6). This tumor antigen, also known as CA9, is a member of the carbonic anhydrase family that is thought to play a role in the regulation of cell proliferation in response to hypoxic conditions and may be involved in oncogenesis and tumor progression (7, 8). Previous studies using a monoclonal antibody against CA9 have shown that CA9 is induced constitutively in certain tumor types but is absent in most normal tissues, with the exception of epithelial cells of the gastric mucosa (4). Furthermore, previous immunobiochemical studies of malignant and benign renal tissues revealed that CA9 was also highly expressed in RCC, suggesting that CA9 expression may be a useful diagnostic biomarker (9, 10). More recently, we reported in a large series of 321 patients that CA9 was expressed in 95% of clear cell carcinoma and higher expression of CA9 is associated with better prognosis of kidney cancer patients, as well as a better response to IL-2 immunotherapy (11).

CA9 contains an epitope that has been shown to be HLAA2.1 restricted and both naturally processed and immunogenic (11). Some tumor-infiltrating lymphocytes lines isolated from kidney tumors are able to react in a CA9-specific manner, although the reaction is weak, as expected from a nonmutated

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<sup>2</sup> The abbreviations used are: RCC, renal cell carcinoma; GMCA-9, granulocyte macrophage colony-stimulating factor and carbonic anhydrase IX; GM-CSF, granulocyte-macrophage colony-stimulating factor; ASGM-1, anti-asialoGM-1; IL, interleukin; DC, dendritic cell; NK, natural killer; UCLA, University of California at Los Angeles; CA9, carbonic anhydrase IX; CMV, cytomegalovirus; LDH, lactate dehydrogenase; m.o.i., multiplicity of infection; SCID, severe compromised immunodeficiency disease; TNF, tumor necrosis factor; PBL, peripheral blood lymphocyte; TCA, trichloroacetic acid; GFP, green fluorescent protein; PE, phycoerythrin.

tissue-specific antigen (13). Despite the immunological properties of CA9 and its selective high expression in kidney cancer, RCC is nonetheless capable of evading the immune surveillance through a variety of local immune suppression mechanisms that have been formidable obstacles to effectiveness of immune stimulation in kidney cancer patients (14). In addition, the poor prognosis of the patients with advanced or metastatic RCC suggests that the CA9 expression is not sufficient to promote a host antitumor response.

In an attempt to enhance the immunogenicity of the CA9 for potential use as an RCC vaccine, we recently reported an innovative approach that combines CA9 and the potent cytokine GM-CSF into a single fusion gene, designated here *GMCA-9*. DCs pulsed with recombinant *GMCA-9* protein *in vitro* were able to induce CA9-specific CTLs (15). The rationale for this approach is based on the fact that GM-CSF has been shown to have clinical activity for RCC (16), and therefore, the addition of GM-CSF to CA9 as a fusion molecule may enhance the immunogenic potential of CA9-based vaccines. In addition, GM-CSF has been shown to be superior to other cytokines in generating a systemic, long-lasting immune response (17) attributable to its critical role for development and differentiation of DCs, the most potent antigen-presenting cells (18–20).

Studies comparing the efficacy of DC-based vaccines have shown that DCs transduced with tumor antigens are superior to peptide-pulsed DCs for eliciting both CD8+ and CD4+ T-cell responses (21). In addition, DCs transduced with recombinant adenoviruses encoding a tumor antigen have been shown to be efficient for *in vitro* CTL generation (22, 23). Adenovirally transduced DCs have the potential to express a variety of tumor antigen epitopes on the surface of DC potentially reducing the problem of antigen loss variants that could evade the immune response (24). Moreover, processing and peptide presentation through endogenous routes are more efficient for cell surface display than exogenous loading of synthetic peptides (21, 25), thereby offering a unique advantage for raising immunity against weak tumor antigens (26), such as CA9 (13). On the other hand, antigen-presenting cells in cancer patients are often functionally impaired, which is likely to reduce the efficacy of protocols dependent on antigen transfer (27).

Here, we report two new approaches for a *GMCA-9*-based RCC vaccine, both based on the intracellular delivery of the fusion protein: (a) an RCC cell line stably transfected with the *GMCA-9* gene; and (b) DCs transduced with a recombinant adenovirus containing the fusion gene. Moreover, we describe the ability of DCs expressing *GMCA-9* to generate CA9-specific CTLs.

## MATERIALS AND METHODS

**Cloning of the *GMCA-9* Gene into a Mammalian Expression Vector.** Generation of a plasmid containing the *GMCA-9* fusion gene, pVL1393/GM-CSF-G250, was described previously (15). The nucleotides coding for the His-Tag were removed by PCR (Expand High Fidelity PCR System; Boehringer Mannheim) using pVL1393/GM-CSF-CA9 as a template and specific forward (5'-TCATGGTACCATGTGGCTGCA-GAGCC-3') and reverse (5'-ATCCTCGAGCTAGGCTC-CAGTCTCGGCTACCTC-3') primers containing a *KpnI* and

*XhoI* site, respectively. The conditions for the reaction were: 3' at 95°C (1×), 40' at 95°C, 30' at 60°C, 2' at 72°C (10×), 40' at 95°C, 30' at 60°C, 2' at 72°C (25×, with additional extension of 5" per cycle), and a final extension of 7' at 72°C. Concentrations of the reactives were recommended by the manufacturer plus 4% of DMSO. PCR product was subcloned in pGEM-T-Easy vector (Promega, Madison, WI), and the resulting plasmid was digested with *KpnI* and *XhoI* and ligated into *KpnI/XhoI*-digested pCEP4 (Invitrogen, Carlsbad, CA), yielding pCEP4-GMCA-9. The fusion gene was finally cloned into the mammalian expression vector pcDNA3.1 after *NotI* digestion of the plasmid and *NotI* partial digestion of pCEP4-GMCA-9, followed by ligation with T4 DNA ligase (Promega). Orientation of the gene was verified with *SalI* digestion. Plasmids were purified using maxipreps and minipreps (Qiagen, Valencia, CA) following standard procedures. To ensure the fidelity of the sequence, the complete insert was sequenced by the UCLA sequencing facility using a set of forward and reverse primers. Enzymes for restriction analysis were obtained from Life Technologies, Inc. (Rockville, MD).

**Cell Lines.** RCC cell lines R11 and R6 were obtained from surgical specimens as described previously (28), cultured in RPMI 1640 (Life Technologies), and supplemented with 10% fetal bovine serum (Life Technologies) and penicillin/streptomycin (50 units/ml each; GEMINI Bio-products, Woodland, CA). Other cell lines used in this study were 293 (embryonic kidney cell line), Daudi (Burkitt's lymphoma cell line), K562 (erythroleukemia cell line), and a cell line stably transfected with the gene encoding CA9 (CA9+) that was kindly provided by Dr. Oosterwijk (13). Embryonic cell line 293 was cultured in DMEM media (Life Technologies, Inc.) and 10% fetal bovine serum plus antibiotics as detailed above.

**Reverse Transcription-PCR Analysis of CA9 Expression.** Total RNA was extracted from different cell lines (1 × 10<sup>6</sup> cells) using acid guanidine isothiocyanate-phenol-chloroform extraction. Reverse transcription of mRNA into cDNA was carried out by incubating titrated RNA with avian myeloblastosis virus reverse transcriptase, primer oligo(dT), deoxynucleotide triphosphate mix, and RNAase inhibitor for 1 h at 42°C. One μl of each cDNA sample was amplified by PCR in a total volume of 25 μl (30 ng of [<sup>32</sup>P]5'-oligonucleotide, 100 ng of 3'-oligonucleotide primer, 2.5 μl of modified 10 × PCR buffer, and 1.25 units of Taq polymerase). The PCR mixture was amplified for 25 cycles in a Thermocycler (Perkin-Elmer, Norwalk, CT). Each cycle consisted of denaturation at 94°C for 1 min and annealing/extension at 65°C for 2 min. The <sup>32</sup>P-labeled products were visualized by autoradiography after acrylamide gel electrophoresis. Amplification of β-actin mRNA was used as an internal control. The sequences of the oligonucleotides are as follows: (a) β-actin (forward, 5'-CAACTCCATCATGAAGTGTGAC-3'; reverse, 5'-CCACACGGAGTACTTGCGCTC-3'); and (b) G250 (forward, 5'-CGGATGCAGGAGGATTCCCCCTGG-3'; reverse, 5'-GACTCTGGTCATCCCCCTTTGTC-3').

**Transfections and Western Blot Analysis.** A CA9-negative RCC cell line, R11, was transfected with pcDNA3.1-GMCA-9 using Effectene (Qiagen) following the manufacturer's protocol and further selected with Neomycin for 3 weeks. Expression of the fusion protein in the stably transfected cell line R11-GMCA-9 was determined by SDS-PAGE and Western

blots. Briefly, cells were harvested after incubation with 10% trypsin (Sigma), washed twice with cold PBS, resuspended in lysis buffer [50 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.5% Triton X-100, 1 mM DTT, and 10% of a protease inhibitor cocktail; Sigma], incubated 15' at room temperature, and centrifuged 10' at  $12000 \times g$ . Supernatants were harvested and quantified for protein concentration using the Bradford assay, and 30  $\mu$ g were electrophoresed on a 12% SDS-PAGE under denaturing conditions. The gel was electrophoretically transferred to a nitrocellulose membrane that was probed with the appropriate primary (anti-CA9 antibody, M75, 1:5000 dilution, kindly provided by Dr. Eric Stanbridge; Ref. 29) or anti-GM-CSF, 1:2000 dilution, purchased from Genzyme Co. (Cambridge, MA), and horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution). Nitrocellulose membranes were then developed using a light-emitting, nonradioactive method (ECL Kit; Amersham Pharmacia Biotech, Buckinghamshire, England) and exposed to films (Fujifilm) for autoradiography.

**Generation of Adenoviruses Containing the GMCA-9 Gene.** The AdEasy System (30) was used for the generation of a recombinant adenovirus containing the *GMCA-9* gene. The plasmid pcDNA3.1-GMCA-9, and the plasmid from the AdEasy system, pAdTrack-CMV (containing the *GFP* gene), were digested using *KpnI* and *XhoI*. The *GMCA-9* gene and digested plasmid were gel purified and ligated obtaining pAdTrack-CMV-GMCA-9. Bacteria were cotransformed with this plasmid and pAdEasy-1 (containing the viral backbone) and selected with Kanamycin (Life Technologies) to isolate the proper recombinants. The complete adenovector was linearized and used for transfection of a mammalian-packaging cell line, 293, where they were further amplified, purified, and titered using GFP-transducing units. The resultant recombinant adenovirus was called AdGMCA-9. Viruses were isolated from 293 cell lysates, and viral DNA was extracted as reported previously (31), digested with *KpnI* and *XhoI*, and electrophoresed in an agarose gel that was stained with ethidium bromide for visualization. The presence of the gene was determined by Southern blot using a digoxigenine-labeled CA9 probe. Generation of the control adenovirus containing the *GFP* gene was reported previously by our group (32).

**DC Generation and Transduction.** DCs were generated from healthy donor's PBL after Ficoll purification. Briefly, whole blood was harvested in EDTA-treated tubes as anticoagulant, further mixed with Ficoll, and centrifuged at  $250 \times g$  for 5 min. The PBL ring was removed with a Pasteur pipette and washed twice with sterile PBS. Cells were resuspended at a concentration of  $7 \times 10^6$  cells/ml in RPMI 1640 (Life Technologies), supplemented with 5% heat-inactivated human AB serum (Omega-Scientific, Tarzana, CA) and antibiotics (penicillin and streptomycin, 50 units/ml each), and then plated in T25 flasks (Corning, Cypress, CA). After an incubation period of 2 h at 37°C, nonadherent cells were removed and frozen for further CTL generation. Fresh DC medium supplemented with GM-CSF (50 ng/ml; R&D Systems) and IL-4 (40 ng/ml; BD Biosciences, Bedford, MA) was added to the adherent cells and incubated for 7 days. Nonadherent cells (immature DCs) were then harvested and plated for further maturation. When needed, TNF- $\alpha$  (100 ng/ml; BD Biosciences) was added to the medium for 48 h to obtain mature DCs.

Human DCs were transduced with AdGMCA-9 at an m.o.i. of 50, and after 24 h, autologous nonadherent PBLs (PBL:DC ratio of 5) were added and incubated 2 weeks in the presence of low concentration IL-2 (40 units/ml).

**Cytotoxicity Assays.** To determine specific cytotoxicity, we used the CytoTox 96 Nonradioactive Cytotoxicity assay (Promega) based on the calorimetric detection of the released enzyme LDH. Target cells were harvested, washed, counted, and diluted to  $5 \times 10^4$  cells/ml, and 50  $\mu$ l/well were plated in a 96  $\times$  well plate. Lymphocytes were washed, counted, diluted, and added at an effector:target cell ratio of 5:1. All of the conditions were assayed in quadruplicate. After 4 h at 37°C, 50  $\mu$ l of supernatants were assayed for LDH activity following the manufacturer's protocol. Controls for spontaneous LDH release in effector and target cells, as well as target maximum release, were prepared. The calculation of cytotoxicity percentage was as follows:

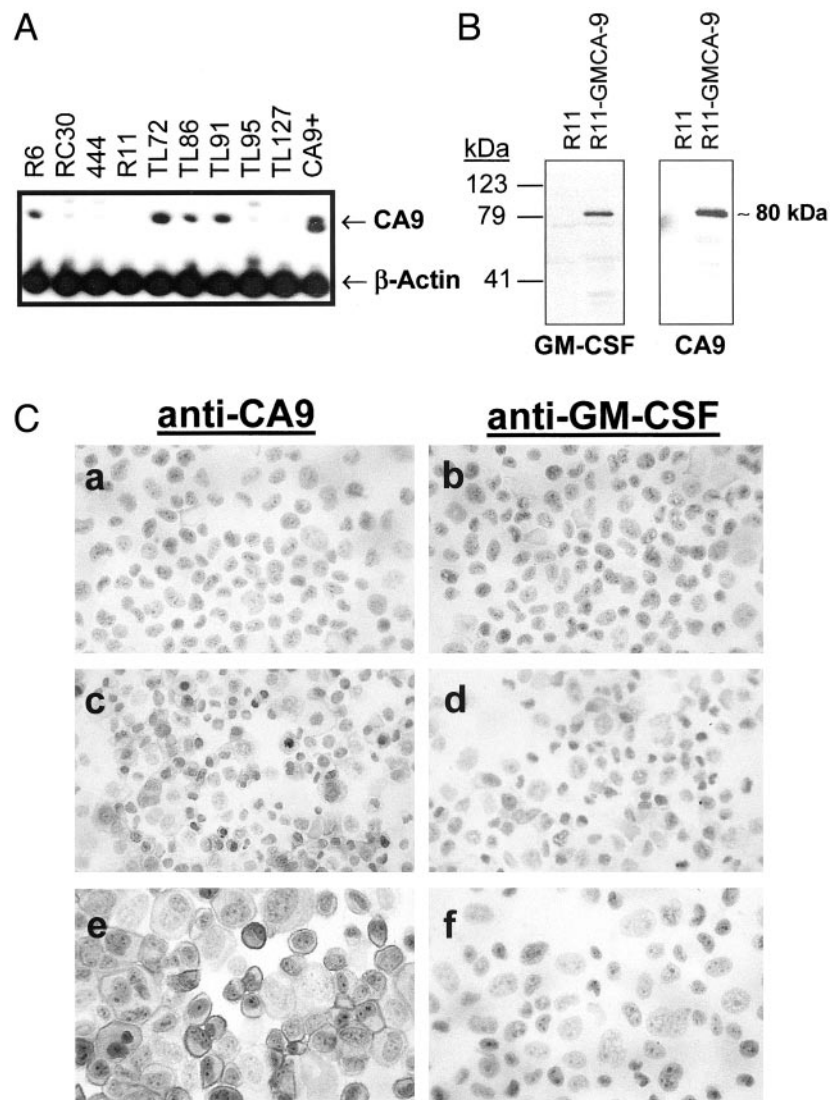
$$\% \text{ cytotoxicity} = \frac{[\text{Experimental} - \text{effector spontaneous}] - [\text{Target spontaneous}]}{[\text{Target maximum} - \text{target spontaneous}]} \times 100$$

Only targets with spontaneous release of LDH  $\leq 10\%$  of the maximum release were considered.

**Immunocytochemistry.** Tumor cells or DCs were harvested, washed, and resuspended in PBS at  $1 \times 10^6$  cells/ml before staining. Briefly, cytopreps were made using Shandon Cytospin 2 (Shandon, Pittsburgh, PA). After air drying, slides were fixed in cold methanol and then cold acetone for 10 min each and rinsed in PBS. Slides were then incubated in 2% BSA for 10 min, and primary antibody [rabbit anti-GM-CSF 1/500, mouse anti-G250 1/700, or mouse IgG1 (DAKO Corp., Carpinteria, CA) 1/200] was added and incubated at 4°C overnight. The following morning, the slides were rinsed in PBS and stained on a DAKO AutoStainer using a 1:1 mixture of peroxidase-conjugated Envision plus anti-mouse or antirabbit immunoglobulins (DAKO) for 30 min and the 3,3'-diaminobenzidine/hydrogen peroxide as the chromogen for 10 min. Finally, slides were counterstained with Harris hematoxylin and methyl green. Monoclonal antibodies G250, specific for CA9 (provided by Dr. Oosterwijk; Ref. 5), and polyclonal anti-GM-CSF (Genzyme) were used for the stainings.

**Animal Studies.** Transgenic male 6–8-week-old mice with SCID were obtained from the breeding program at the UCLA and manipulated under sterile conditions following UCLA-approved protocols. All animals were anesthetized with ketamine and xylazine before inoculation with cancer cells. For the tumorigenicity studies, tumor cells were harvested, washed in sterile PBS, counted, and inoculated ( $5 \times 10^6$  cells/300  $\mu$ l of PBS) s.c. in the right flank. For the functional depletion of NK cells, SCID mice were injected with 200  $\mu$ l of a 1:10 dilution in PBS of the original stock of the antibody ASGM-1 (Wako Chemicals, Richmond, VA), 3 days before the tumor challenge and then on a weekly basis. Mice were sacrificed when tumor diameters reached 10 mm, according to UCLA policies. Each experimental condition consisted of four mice per group.

**ELISA.** For the determination of GM-CSF concentration,  $3 \times 10^5$  PBLs or 293 cells were seeded in a 6  $\times$  well plate with 5 ml of complete RPMI medium. PBLs were incubated with low concentration of IL-2 (50 ng/ml). 293 cells were infected with



**Fig. 1** Expression of GMCA-9 in an RCC cell line. **A**, CA9 mRNA expression in different RCC cell lines determined by reverse transcription-PCR. A cell line stably transfected with the CA9 gene was used as a positive control (CA9+). **B**, Western blots for GMCA-9 in R11 control (mock-transfected) and R11-GMCA-9 using GM-CSF or CA9 antibodies. **C**, immunocytochemical stainings for GMCA-9 using anti-CA9 (left panels) or anti-GM-CSF antibodies (right panels) of R11 control (a and b), R11-GMCA-9 (c and d), and a CA9-positive RCC cell line R6 (e and f).

AdGMCA-9 at an m.o.i. of 50:1 for 24 h. Cell culture supernatants were harvested and centrifuged at  $250 \times g$  to remove cell debris and assayed for GM-CSF by ELISA using the Quantikine Kit (R&D Systems) according to the manufacturer's instructions. Briefly, wells coated with a murine monoclonal antibody against human GM-CSF were blocked with a buffered protein assay diluent, and after extensive washing, 100  $\mu$ l of supernatant were added in duplicate, incubated for 2 h at room temperature, and thoroughly washed to remove unbound substances. A horseradish peroxidase-linked antibody against GM-CSF was then added and developed using a substrate solution. After stopping the reaction, absorbance at 450 nm was measured for each well and compared with the GM-CSF standard curve.

**Flow Cytometry.** Samples of  $1 \times 10^5$  DCs or PBLs were harvested, washed, and resuspended in 50  $\mu$ l of PBS. An additional 50  $\mu$ l of PBS containing 5  $\mu$ l of the FITC- and/or PE-conjugated antibody were added per reaction. After incubation at 4°C for 30 min, cells were washed with PBS, resus-

pended in 300  $\mu$ l of buffer, and analyzed on a Becton Dickinson FACScan flow cytometer that simultaneously acquires forward and side scatters as well as FL1 (FITC) and FL2 (PE) data. Processing of the data were carried out with CellQuest Software (Becton Dickinson, San Jose, CA). The following antibodies were used for the characterization of DC and PBL phenotype: (a) CD4; (b) CD8; (c) CD56; (d) TcR; (e) CD83 (all purchased from Becton Dickinson); and (f) HLA DR (Immunotech)

**Statistical Analysis.** Data are represented as mean values  $\pm$  SD. Statistical significance was analyzed using Student's *t* test, and a probability value of  $\leq 0.05$  was considered significant (Excel; Microsoft, Redmond, WA).

## RESULTS

**Generation of a Universal RCC Cell Line Expressing the GMCA-9 Fusion Protein.** A panel of RCC cell lines generated in our laboratory was tested for CA9 mRNA expres-

sion (Fig. 1A). A CA9-negative cell line, R11, was stably transfected with pcDNA3.1-GMCA-9 generating the transfectant R11-GMCA-9. Expression of the fusion protein GMCA-9 in the transfectant was assessed by Western blot analysis after denaturing SDS-PAGE and also by immunocytochemistry using monoclonal antibodies for CA9 and GM-CSF. In Western blots, the fusion protein appears as an  $M_r$  80,000 band when stained either for CA9 or GM-CSF (Fig. 1B). The immunocytochemical study (Fig. 1C) with CA9 antibody demonstrates a cell membrane distribution pattern of the GMCA-9 fusion protein in addition to some cytoplasmic staining. Although a codistribution of the CA9 and GM-CSF stainings was expected, GM-CSF staining was less pronounced and with a cytoplasmic pattern of distribution.

**GMCA-9 Inhibits Tumor Growth of a RCC Cell Line in SCID Mice.** An RCC cell line expressing the fusion protein R11-GMCA-9 did not exhibit any significant change in the *in vitro* growth when compared with the same cell line mock transfected, R11-control (Fig. 2A). We then examined the *in vivo* effect of the GMCA-9 expression. Previous studies demonstrated that the RCC cell line R11 is tumorigenic in SCID mice (28). We elected to inject SCID mice *s.c.* with the transfected cell line R11-GMCA-9 and the mock-transfected control cell line R11-control. After 4 weeks, tumors were growing significantly slower in mice injected with R11-GMCA-9 (Fig. 2B) than in those injected with the control cell line (week 5,  $1 \pm 1.15$  versus  $10.25 \pm 2.06$ ,  $P < 0.01$ ), suggesting that the expression of the fusion protein GMCA-9 is associated with a significant inhibition in the tumor growth of the RCC cell line R11 in SCID mice. These *in vivo* studies were reproducible, and the same tumor growth inhibition was observed in two additional experiments ( $n = 5$  and  $4$ /group; data not shown).

**Role of NK Cells in the *In Vivo* Inhibition of Tumorigenicity of R11-GMCA-9 Cell Line.** SCID mice are partially immunodeficient and lack B and T cells but still have functional NK cells, macrophages, and DCs (Langerhans cells). To determine the role of NK cells in the inhibition of tumorigenicity, R11-GMCA-9 and R11-control cells were injected in mice control as well as in mice treated with the antibody anti-asialo GM-1 (anti-ASGM-1) that has been demonstrated to inhibit NK function in mice (33). Tumor growth of RCC-control line was similar in the untreated and NK-depleted SCID mice (Fig. 2C). In contrast, R11-GMCA-9 tumors grew significantly faster in NK-depleted SCID mice compared with control SCID (week 5,  $5 \pm 1.41$  versus  $1 \pm 1.15$ ,  $P < 0.05$ ), although their growth was slower than R11-control tumors, suggesting that NK cells are at least partially involved in the inhibition of tumorigenesis in these mice.

**Characterization of a Recombinant Adenovirus Expressing the GMCA-9 Fusion Gene.** A replication-deficient, E1- and E3-deleted, recombinant adenovirus containing the fusion gene GMCA-9 (AdGMCA-9) was used to express the GMCA-9 protein. Confirmation of the intact GMCA-9 gene was determined by restriction analysis (Fig. 3A, a) and Southern blot analysis (Fig. 3A, b) of the purified recombinant viral DNA. The expression and cellular location of GMCA-9 protein in 293 cells infected with the AdGMCA-9 were determined by Western blot and immunocytochemistry. By Western blot, an additional band at  $M_r$  85,000 was identified in 293 cells that were transfected

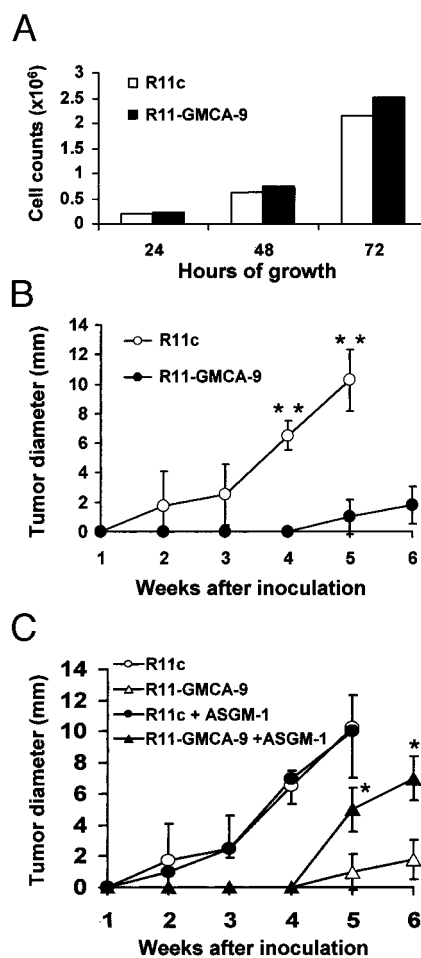
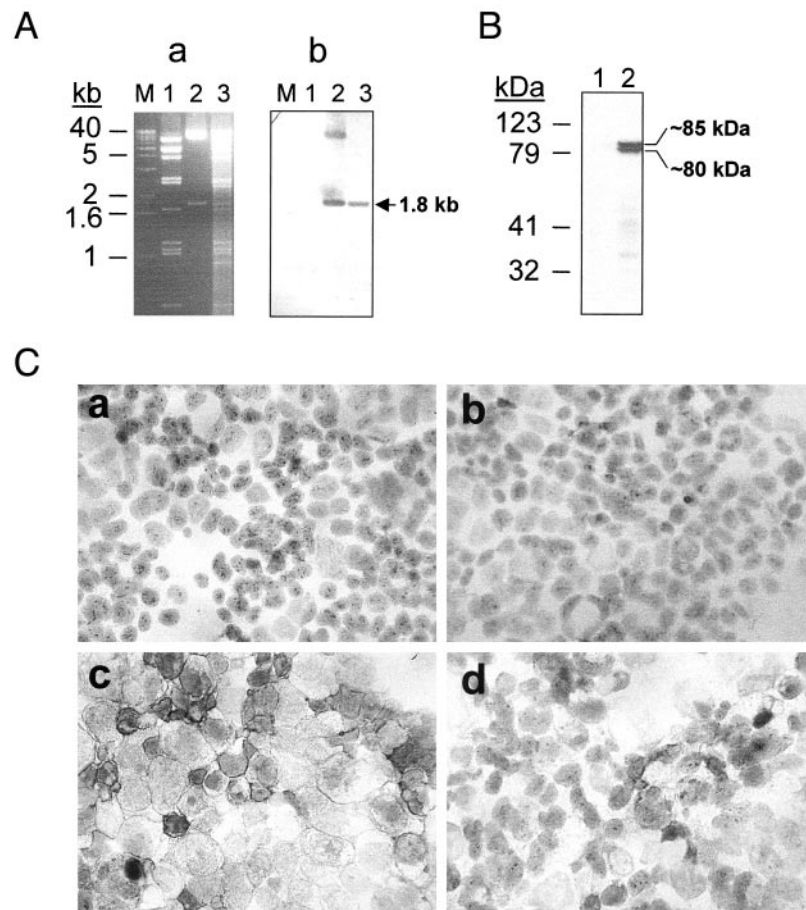


Fig. 2 Effect of the GMCA-9 expression on the *in vitro* and *in vivo* growth of the RCC cell line R11. A, comparison of the *in vitro* growth of RCC cell line control R11 (mock transfected) and R11-GMCA-9. No statistically significant differences were found. B, tumorigenicity of these cell lines in SCID mice ( $n = 4$ ). C, effect of the NK-blocking agent ASGM-1 on the tumorigenicity of R11 control and R11-GMCA-9. \* $P < 0.05$ , \*\* $P < 0.01$  by Student's *t* test.

with AdGMCA-9 (Fig. 3B), which was not present in the R11-GMCA-9 cell line that had stable expression of GMCA-9. Immunocytochemical analysis of 293 cells infected with adenogMCA-9 (Fig. 3C) exhibited a similar pattern of cell membrane distribution compared with R11-GMCA-9 but with higher amounts of GMCA-9 protein confirming that AdGMCA-9 was providing stronger expression of the fusion gene.

**Transduction with AdGMCA-9 Produces Secretory GM-CSF.** Construction of the GMCA-9 fusion gene involved combining the full-length coding regions of both the GM-CSF and CA9 genes, including the CA9 signal peptide. Therefore, despite the protein appearance as a well-defined single or double band in the Western blots of transfected or infected cells, respectively, processing of the fusion protein was still a possibility. To determine the presence of the GM-CSF, normally a secreted cytokine, produced by our GMCA-9 construct, we precipitated proteins from culture supernatants using a standard



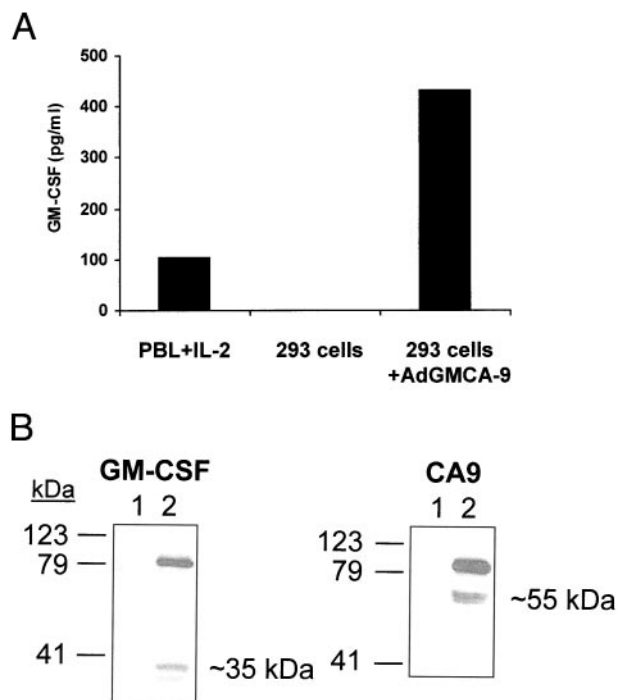
**Fig. 3** Generation of a replication-defective adenovirus containing the *GMCA-9* gene. **A**, (a), agarose gel of *KpnI* + *XhoI* restriction digestions of: 1, AdEasy plasmid as a negative control; 2, pCEP-4-GMCA-9; and 3, viral DNA purified from Ad GMCA-9-infected cells. **b**, Southern blot of this agarose gel, incubated with a digoxigenin-labeled CA9 probe, revealing the presence of the *GMCA-9* gene (1.8-kb band). In **B**, Western blot for GMCA-9 protein was incubated with anti-GM-CSF antibody. 1, 293 cells; 2, 293 cells infected with AdGMCA-9. **C**, immunocytochemical staining of 293 cells alone (a and b) or infected with AdGMCA-9 (c and d) using anti-CA9 (left panels) or anti-GM-CSF (right panels).

TCA precipitation protocol but failed to identify a GM-CSF product. However, using a GM-CSF ELISA Kit capable of detecting concentrations as low as picograms per milliliter, we found significant amounts of GM-CSF in the AdGMCA-9-infected 293 cells and none in noninfected cells (Fig. 4A). The result suggested that some of the GMCA-9 fusion protein was proteolytically processed into both GM-CSF and CA9 moieties. Considering the number of cells, volume of medium, and time of incubation (see "Materials and Methods"), the amount of secreted GM-CSF was determined to be 7.3 ng/10<sup>6</sup> cells/24 h. Viability of the cells after harvesting was determined to be close to 100%, thereby precluding the possibility that the GM-CSF detected was from GMCA-9 protein released from dead cells.

We then further investigated the presence of the partially processed GMCA-9 in Western blots of cell pellets of the transfectant R11-GMCA-9 and could observe the presence of GM-CSF- and CA9-like products when incubated with anti-GM-CSF (Fig. 4B) or anti-CA9 (Fig. 4C), respectively. Considering the molecular weight of  $M_r$  80–85,000 for the fusion protein, molecular weights for the partially processed GMCA-9 were found to be in the expected size, at  $M_r$  ~54–58,000 for CA9 and  $M_r$  30,000 for the GM-CSF-like product. The amount of processed protein was determined from Fig. 4C to be ~30%.

**Expression of GMCA-9 in Human DCs Transduced with AdGMCA-9.** Human immature DCs were generated from PBLs in the presence of GM-CSF and IL-4 for 7 days as described in "Materials and Methods." These DCs were subsequently harvested and transduced with adenoGMCA-9 at an m.o.i. of 50. After 24 h of incubation, GFP expression was determined by fluorescence microscopy showing a 100% efficiency of infection (Fig. 5, A and B). Cells were detached, washed twice with PBS, and further stained with anti-CA9 (Fig. 5C) or anti-GM-CSF (Fig. 5D), revealing the presence of high amounts of the fusion protein GMCA-9 in the cell membrane, comparable with CA9-expressing RCC cell lines (R6; Fig. 1e). An adenovirus containing the *GFP* gene driven by the CMV promoter was used as a control for staining (Fig. 5, E and F). Interestingly, DCs transduced with the control adenovirus were also positively stained for GM-CSF, albeit with lower intensity, suggesting that some of the GM-CSF used for the DC generation was still present in the surface of these DCs after 24 h.

**GMCA-9 Is Capable of Inducing DC Maturation.** Previous experiments have shown that the purified GMCA-9 protein is able to induce DC differentiation, when combined with IL-4, and also induce further DC maturation (15). The phenotype of the DCs was assessed by flow cytometry after transduc-



**Fig. 4** Partial processing of GMCA-9 protein and delivery of GM-CSF. **A**, GM-CSF ELISA of cell culture supernatants of PBLs stimulated with low dose IL-2 (50 ng/ml) as a positive control, 293 cells alone, and infected with Ad GMCA-9. No CPE was present in infected cells. In **B**, Western blots for GMCA-9 of R11 (Lane 1) and R11-GMCA-9 cells (Lane 2) were revealed using anti-GM-CSF or anti-CA9 antibodies, revealing in each case the presence of the fusion protein ( $M_r$  ~80,000) plus two bands with molecular weights corresponding to a GM-CSF-like product ( $M_r$  ~35,000), when stained with anti-GM-CSF, and CA9 ( $M_r$  ~55,000), when stained with antiCA9 antibodies.

tion with AdGMCA-9 to determine the ability of GMCA-9 to induce DC maturation. DCs transduced with Ad-GMCA-9 exhibited a strong up-regulation of HLA-DR expression compared with control adenovirus, as well as TNF- $\alpha$ -matured DCs (Table 1). In addition, up-regulation of the maturation marker CD83 was also observed in transduced DCs when compared with the controls. Together, these results suggest that GMCA-9 protein alone expressed inside the DCs is capable of inducing maturation of these cells and up-regulate the antigen presentation machinery.

**CA9-specific CTL Induction by AdGMCA-9-transduced DCs.** Although induction of PBL proliferation was determined to be lower for PBLs stimulated in the presence of DC cells transduced with the AdGMCA-9 (Fig. 6A), this specific group of PBLs demonstrated a significant up-regulation of T-cell receptor expression, compared with PBLs stimulated with control DCs. The percentages of CD4+CD8 $^-$ , CD56+TcR $^-$ , and CD56+TcR $^+$  were also higher in PBLs stimulated with AdGMCA-9-transduced DCs (Fig. 6B), suggesting an increase in the amount of T-helper cells, as well as NK-T cells.

To determine the ability of DCs transduced with AdGMCA-9 to generate CA9-specific CTLs, cytotoxicity was assessed using a CA9-negative cell line, R11, and a CA9-positive cell line, CA9+ (13). As shown in Fig. 6C, a CA9-

specific cytotoxicity was shown only in the case of PBLs incubated with DCs transduced with AdGMCA-9, suggesting that GMCA-9-transduced DCs are able to stimulate the generation of CA9-specific CTLs *in vitro*.

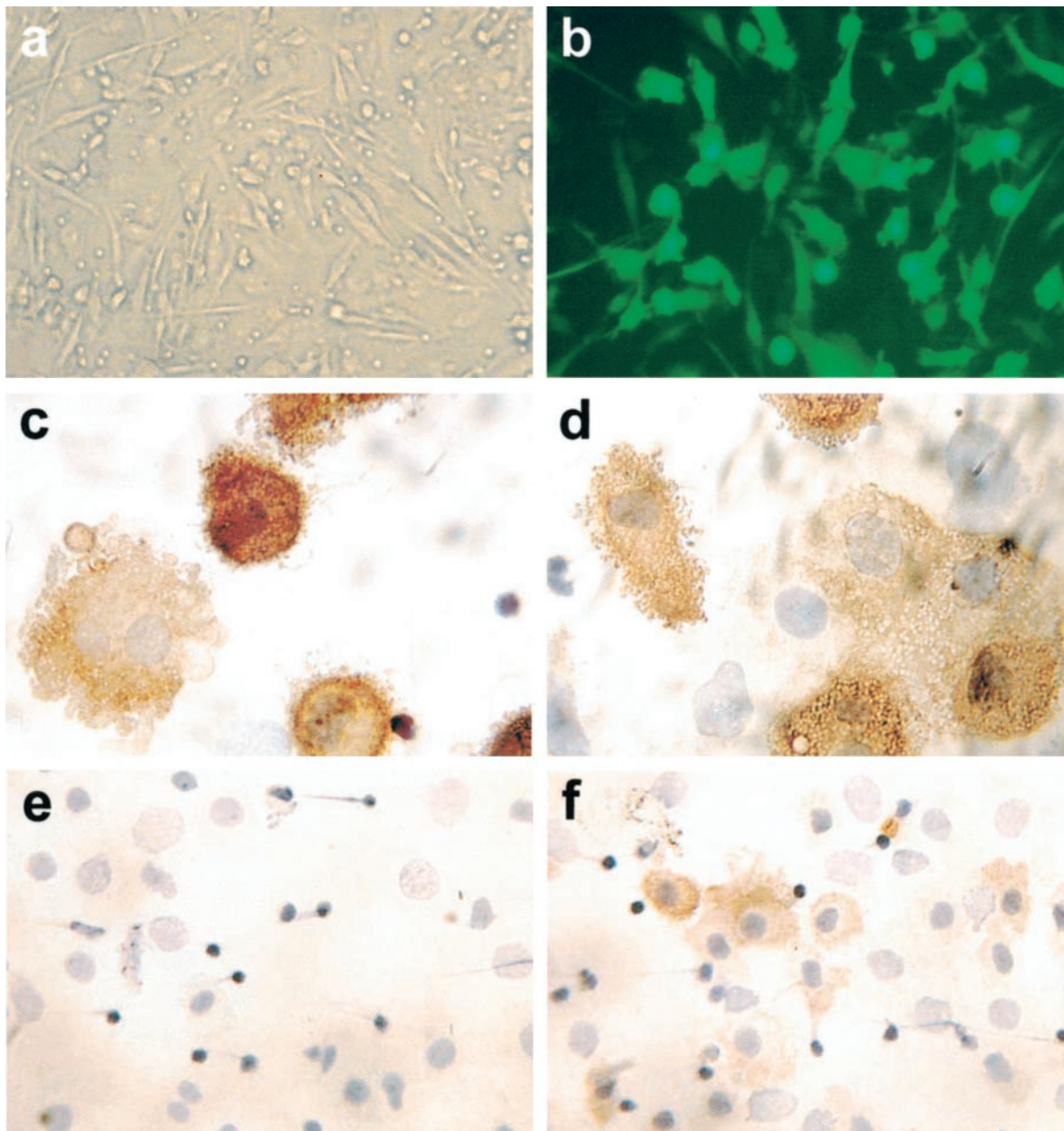
## DISCUSSION

We have shown previously that DCs pulsed with the purified recombinant GMCA-9 protein are capable of differentiation and maturation in the presence of IL-4 without the addition of exogenous GM-CSF. Moreover, these GMCA-9-pulsed DCs generated CA9-specific CTLs that included both CD8 $^+$  and CD4 $^+$  T cells (15).

Here, we report two new approaches for the characterization of the *in vitro*, as well as *in vivo*, immunomodulatory capabilities of this fusion protein in an attempt to establish its utility as an GMCA-9-based RCC vaccine. Both approaches are based on the intracellular delivery of the protein: (a) a RCC cell line stably transfected with the GMCA-9 gene; and (b) DCs transduced with a recombinant adenovirus containing the fusion gene.

Our studies of the GMCA-9 expression in the RCC stably transfected cell line show that the fusion protein is synthesized as a polypeptide of  $M_r$  80,000. This is in contrast with the theoretical size of  $M_r$  66,000, the same size reported previously for the GMCA-9 protein when obtained using a baculoviral system in insect cells (15), suggesting that when expressed inside the cell, our fusion protein GMCA-9 undergoes post-translational modification. This is likely attributable to glycosylation as has been reported for each one of its components, GM-CSF (34, 35) and CA9 (29). Moreover, defective post-translational modifications have been reported in insect cells, which often results in lower levels of glycosylation and/or phosphorylation of the target protein (36, 37). Interestingly, in Western blots of AdGMCA-9-infected cells, GMCA-9 appears as a doublet. Similar variations in the pattern of the protein in Western blots have been reported for CA9, depending on the cell line studied (6). Although the immunocytochemical stainings with the CA9 antibodies together with the results in Western blots sufficiently demonstrate that the GMCA-9 is expressed in a stable way and mainly located in the cell membrane, stainings with anti-GM-CSF showed a weak cytoplasm-associated pattern, most likely caused by the partial processing of the protein and secretion of a GM-CSF-like product, as suggested by the detailed study of Western blots and cell culture supernatants of the AdGMCA-9-infected cells. Partial processing of the fusion protein, with the subsequent secretion of a GM-CSF-like product, is especially interesting, because it could establish a gradient that could enhance the potency of this vaccine, particularly in the case of transduced DCs. Recently, similar protein processing in GM-CSF-containing fusion proteins engineered to be bound to the cell membrane has been reported (38).

*Ex vivo* culture of DCs can restore their immunostimulatory functions, circumventing tumor-induced impairment of antigen presentation (18). Thus, a recombinant replication-defective adenovirus containing the GMCA-9 fusion gene was generated for the *ex vivo* transduction of DCs as an approach for a GMCA-9-based kidney cancer vaccine. Transduced DCs produce high amounts of GMCA-9 protein only 24 h after the



**Fig. 5** Expression of *GMCA-9* in human DCs transduced with Ad*GMCA-9*. Human immature DCs generated from PBLs in the presence of IL-4 plus GM-CSF were transduced at an m.o.i. of 50:1 with Ad*GMCA-9*, incubated for 24 h, and visualized using a phase contrast microscope under normal light (a) or UV light (b), revealing the presence of the virus that contains the *GFP* gene in addition to *GMCA-9*. DCs transduced with an adenovirus (AdPSE-SR39) containing also the *GFP* gene were used as a control (e and f). DCs were then harvested, washed with PBS, and stained using anti-CA9 (c and e) or anti-GM-CSF (d and f).

transduction and express the protein at the cell membrane. Moreover, the fusion protein is able to induce DC maturation as shown by the significant increase of CD83<sup>+</sup> and HLA-DR<sup>+</sup> in Ad*GMCA-9*-transduced DCs that could be attributable either to the processing of the *GMCA-9* by the DC and/or to the presence of the GM-CSF part of the fusion protein in the cell surface and further binding to the GM-CSF receptor.

Using a cell line stably transfected with the *CA9* gene, we determined the *GMCA-9*-mediated, CA9-specific cytolytic ac-

tivity of CTLs generated from PBLs. Study of the phenotype of these CTLs shows the priming of CD4<sup>+</sup>CD8<sup>-</sup>, CD56<sup>+</sup>TcR<sup>-</sup>, and CD56<sup>+</sup>TcR<sup>+</sup> in PBLs stimulated with Ad*GMCA-9*-transduced DCs in the presence of low concentrations of IL-2, suggesting an involvement of T-helper and NK cells, as well as the recently characterized NK-T cells (39, 40). If this is the case, involvement of the T-helper cells is especially interesting, because although most tumor vaccines are designed to maximize the CTL response, new evidence points to the central role of

Table 1 Maturation markers in dendritic cells transduced with AdGMCA-9<sup>a</sup>

	Control <sup>b</sup>	TNF- $\alpha$	AdPSE-SR39	AdGMCA-9
CD83	5.2	5.4	5.7	18.3
HLA-DR	222	204	382	1012

<sup>a</sup> Numbers represent the mean fluorescence intensity, MFI.

<sup>b</sup> Control condition consisted in iDCs incubated with RPMI media alone. TNF- $\alpha$  was added at 500 ng/ml; adenovirus control (*AdPSE-SR39*) and *AdGMCA-9* were added at an m.o.i. of 50:1. All the experiments were carried out in duplicate.

CD4<sup>+</sup> T cells in directing both innate and adaptative antitumor immune responses (14, 41–43), being particularly important for the development of cancer vaccines in kidney cancer because CD4<sup>+</sup> tumor-infiltrating lymphocytes with antitumor reactivity have been isolated from RCCs (44). Recent findings show that the CA9 epitope presented through MHC class I molecules is also effective in stimulating T-helper cells through MHC class II molecules, which is essential for an effective immunotherapy (45).

We decided to test the *in vivo* effects of GMCA-9 RCC in SCID mice that, despite defective T- and B-cell development, still have intact NK cells, macrophages, and Langerhans cells. More importantly, it has been shown that NK cells are cytotoxic for tumor cells in mice (46), and this cytotoxicity is enhanced by injection of human GM-CSF (47). Similarly, Langerhans cells, which are DCs located in the skin epithelium (48), are modulated by GM-CSF and TNF- $\alpha$  (49) and are able to mature and migrate to the lymph nodes, even in the absence of T cells (50). The apparent decreased tumorigenicity of the RCC cell line that constitutively expresses GMCA-9 *in vivo* suggests a possible role of NK cells in mediating this antitumor response. In contrast, CA9 alone does not appear to be slowing the growth of tumors, because RCC cell lines which constitutively express CA9 still grow aggressively in SCID mice. Therefore, we believe that the GM-CSF moiety of GMCA-9 fusion protein is responsible for slowing tumor growth. Whether this effect is attributable to the presence of the GM-CSF in the membrane of the cells, to the GM-CSF delivery after the protein processing, or both remains to be elucidated. The fact that the amount of GM-CSF secreted by our vaccine is about five times lower than the reported threshold for an effective vaccine (51) is suggesting that the GM-CSF moiety of the fusion protein attached to the membrane could be essential in our model. These *in vivo* studies agree with previous reports (52, 53) in suggesting that human GM-CSF has the ability to bind murine GM-CSF receptors *in vivo* and generate a response despite the fact that GM-CSFs from human and murine fail to cross-react in their colony-forming assays *in vitro* (54).

In conclusion, our results show that transduction of the fusion protein GMCA-9 into DCs using an adenoviral vector generates a CA9-specific immune response *in vitro*. In addition, our studies in mice suggest a role for the GM-CSF moiety of the fusion protein *in vivo*, suggesting that GMCA-9 could be a promising molecule for an RCC vaccine. Additional *in vivo* models of DC-mediated stimulation of immune effector cells by GMCA-9 will be important in demonstrating

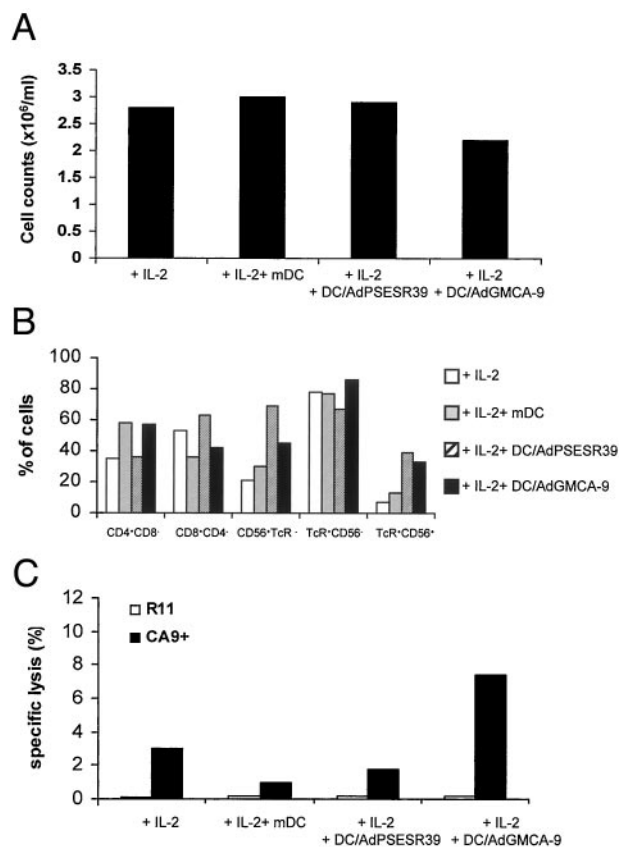


Fig. 6 Growth, phenotype, and cytotoxicity profiles of healthy donor's PBLs stimulated with autologous DCs transduced with AdGMCA-9. A, growth expansion of PBLs using four different strategies: (a) low dose IL-2 (50 ng/ml); (b) IL-2 plus DCs matured with TNF- $\alpha$  (500 ng/ml); (c) IL-2 plus DCs transduced with the adenovirus control; and (d) IL-2 plus DCs transduced with Ad GMCA-9. PBLs ( $3 \times 10^5$ /ml, 3:1 of PBL:DC) were stimulated for 14 days, adding fresh IL-2 every 4 days, and on day 7, PBLs were restimulated with fresh mature or transduced DCs ( $1 \times 10^5$ /ml). B, phenotype of PBLs stimulated with the conditions described above.  $1 \times 10^5$  cells were harvested and incubated with the indicated PE- and FITC-conjugated antibodies, and the fluorescence was determined by flow cytometry as described in "Materials and Methods." C, cytotoxicity of PBLs against heterologous cell lines. GMCA-9-induced, CA9-specific cytotoxicity was determined using CA9-positive (CA9+ cell line, provided by Dr. Oosterwijk) or CA9-negative (RCC cell line R11) targets. Activated PBLs were incubated with target cells ( $5 \times 10^3$ ) at a 5:1 ratio for 4 h, and supernatants were harvested for determination of LDH activity. Lytic units were calculated as described in "Materials and Methods."

tumor treatment and prevention for future clinical trials in patients with advanced RCC.

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