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Adenovirus Infection Enhances Dendritic Cell Immunostimulatory Properties and Induces Natural Killer and T-Cell-mediated Tumor Protection[1]

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ABSTRACT

Dendritic cells (DCs) are potent antigen-presenting cells with the potential for cancer immunotherapy. Adenoviral-mediated gene transfer is an attractive means to manipulate the immunostimulatory properties of DCs for therapeutic advantage. Because adenovirus induces DC maturation, we postulated that it would significantly alter their immune functions. Infected DCs markedly increased allogeneic and antigen-specific T-cell proliferation, and augmented natural killer cell lytic activity and IFN-γ production. The enhanced effector cell stimulation by infected DCs was dependent on their secretion of interleukin 12. Immunization with infected DCs pulsed with tumor antigen protected against flank tumors in 78% of mice and induced a memory response. Antitumor immunity was dependent on both T cells and natural killer cells. Antigen-pulsed, mock-infected DCs were nonprotective. The findings that adenoviral vectors alone critically alter DC immune functions and antitumor properties have important implications for the design and interpretation of immunotherapy regimens using vector-based gene transfer to modulate immunity.

INTRODUCTION

DCs[3] are specialized antigen-presenting cells that initiate immune responses. DCs are commonly generated from bone marrow or isolated from the peripheral blood or spleen. DCs are characterized by their functional and phenotypic properties, and may be classified into two broad categories. Immature DCs are efficient in antigen uptake and are characterized by low surface expression of MHC class I and II proteins, integrins (CD54), and costimulatory molecules (CD40, CD80, and CD86; Ref. 1). Conversely, mature DCs specialize in antigen presentation and possess high surface expression of these markers. DCs have demonstrated potent antitumor properties in a variety of experimental models (2–4). Preliminary clinical trials using DC immunotherapy in the treatment of human cancer have shown some promising results (5, 6).

Gene transfer is an attractive means to manipulate the function of DCs and their interactions with effector cells. A variety of vectors are available for gene transfer to DCs, and recombinant adenovirus is among the most efficient (7). Previous investigations have focused on transferring genes encoding activating cytokines or tumor antigen to DCs. A number of reports have shown DCs to possess enhanced antitumor properties after adenoviral transfer of therapeutic transgenes. For example, DCs overexpressing IL-12 as a result of adenoviral-mediated IL-12 gene transfer have been found to induce antitumor immunity when injected directly into tumors (8, 9). However, whereas the effects of a variety of transgenes on DC function have been described, the impact of the adenoviral vector itself on DC function has not been thoroughly investigated. Such evaluation is critical to accurately interpret the impact of transferring a therapeutic gene.

Initial studies looking specifically at the effect of adenovirus on DC phenotype and cytokine expression have shown that adenovirus matures DCs and induces modest up-regulation of IL-12 expression (10, 11). Therefore, we postulated that AdDCs without a therapeutic transgene would have enhanced immunostimulatory properties. We show that adenoviral induced maturation of DCs alters their ability to capture antigen and to undergo chemotaxis. Furthermore, adenovirus infection alone profoundly enhances DC ability to activate NK cells and T cells. This is shown to be contingent on IL-12 secretion. Finally, we show that immunization with AdDC, without the benefit of a therapeutic transgene, stimulates antitumor immunity *in vivo*. By showing significant effects of the adenovirus vector alone on DC function, these experiments form a vital baseline for present and future investigations using adenoviral gene transfer to DCs. Furthermore, these findings may have important implications to other systems using vector-based gene transfer to modulate immunity.

MATERIALS AND METHODS

Animal Procedures. Male C57Bl/6 mice (3–8 weeks of age) were purchased from Taconic Farms (Germantown, NY). All of the procedures were approved by the Institutional Animal Care and Use Committee. Mice were immunized with two i.p. injections of 5×10^5 DCs given 7 days apart. DCs were either infected overnight with recombinant adenovirus or mock infected. In some cases, DCs were loaded (10 μg/ml for 2 h) with Ova from ovalbumin or βgal from β-galactosidase (Peptide Synthesis Core, Sloan-Kettering Institute). One week later, mice were given a s.c. flank injection of 3.5×10^5 EL-4 lymphoma cells, 3.5×10^5 EG-7 cells (EL-4 cells carrying the chicken ovalbumin gene), 1×10^5 B16 melanoma cells (all ATCC, Rockville, MD), or 2.5×10^5 Pan02 pancreatic tumor cells (National Cancer Institute, Frederick, MD). Depletion studies were performed using three doses of 0.25 mg of GK1.5 (anti-$CD4^+$ T cell), 53–6.72 (anti-$CD8^+$ T cell), or PK136 (anti-NK cell) antibody during the 5 days before DC immunization and then once weekly for up to 6 weeks.

DC Generation and Infection. DCs were isolated by the method of Steinman with modification (12, 13). Bone marrow from C57Bl/6 mice was grown in RPMI 1640 with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.05 mM 2-mercaptoethanol, and GM-CSF (1:30 dilution of J558 supernatant, gift of Ralph Steinman, Rockefeller University, New York, NY). DC were infected with adenovirus on day 8 of culture at a MOI of 500, unless indicated otherwise. Flow cytometry was performed on an EPICS-XL flow cytometer (Beckman Coulter, Fullerton, CA) using antibodies for: MHC class I ($H\text{-}2K^b$) and class II ($I\text{-}A^b$); DC integrin marker CD11c; CD54 (ICAM-1); costimulatory molecules CD40, CD80 (B7–1), and CD86 (B7–2); B cells (CD45R), NK cells (NK1.1), and T cells (CD4 and CD8; PharMingen, San Diego, CA).

Recombinant Adenovirus and Reagents. Recombinant adenoviruses were propagated, purified, and stored as described previously (14). AdGFP encodes GFP under the control of the cytomegalovirus promoter (Quantum Biotechnologies, Montreal, Quebec, Canada). AdAlkPhos (14) contains the alkaline phosphatase reporter gene. Endotoxin was undetectable in viral stocks using the Limulus Amebocyte Lysate Clot Test (sensitivity 6 pg/ml; Associates of Cape Cod, Woods Hole, ME). LPS (Sigma, St. Louis, MO), TNF-α (R&D Systems, Minneapolis, MN), and anti-CD40 obtained from the clone FGK45 (Ref. 15; Monoclonal Antibody Core Facility, Memorial Sloan-Kettering Cancer Center, NY) were used at doses of 1–100 ng/ml.

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[3] The abbreviations used are: DC, dendritic cell; AdDC, dendritic cells infected with adenovirus; Ova, $Ova_{257-264}$ peptide; βgal, $\beta gal_{876-884}$ peptide; MOI, multiplicity of infection; MLR, mixed lymphocyte reaction; IL, interleukin; NK, natural killer; ATCC, American Type Culture Collection; GM-CSF, granulocyte macrophage colony-stimulating factor; LPS, lipopolysaccharide; TNF, tumor necrosis factor; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorter.

Cytokine Measurement. IL-12 (p70), IL-2, IL-4, TNF-α, IFN-γ, and GM-CSF were measured by ELISA (PharMingen). IL-12 was also measured in a bioassay using dilutions of DC supernatant on 1×10^4 2D6 cells (16) in 96-well plates. After 48 h, cells were pulsed with 0.5 μCi/well of thymidine (New England Nuclear, Boston, MA) for 24 h, and tritium incorporation was measured. IL-12 blocking antibody was obtained from the supernatant of R2–10F6 cells (Ref. 17; ATCC) and used at a 1:5 dilution.

Antigen Uptake and Chemotaxis Assays. DCs (5×10^5) were incubated with either FITC-albumin or FITC-mannose albumin (1 mg/ml; Sigma) for 1 h at 37 degrees in 150 μl. Afterward, the percentage of fluorescent cells was determined by flow cytometry. DC chemotaxis was assessed as described with slight modification (18). Briefly, 2.5×10^5 ^{51}Cr-labeled DCs were plated above a 5.0-μm insert (Costar, Corning, NY) in a 24-well dish containing a 600-μl solution of Rantes (500 ng/ml; R&D Systems). After 2 h, migrated cells were collected, lysed using 2% Triton-X, and measured in a gamma counter. Results are expressed as: Migrational Index = (cells migrated to Rantes)/(cells migrated to medium).

T-Cell Proliferation Assays. For MLRs, GFP-positive DCs were sorted by flow cytometry after infection with AdGFP. DCs were then irradiated (3000 rads) and added at various amounts to 3×10^5 syngeneic or allogeneic T lymphocytes in 96-well plates before pulsing with thymidine (1 μCi/well) on day 3 for 20 h. For antigen-specific T-cell-stimulation assays, an H-2K^b-restricted CD8$^+$ T-cell hybridoma specific for Ova was used (19). DCs were incubated with either Ova (10 μg/ml) or Ovalbumin protein (2 mg/ml) overnight and then plated at various concentrations with 5×10^4 Ova-restricted T cells in a 96-well dish for 2 days. Afterward, supernatant was assayed for IL-2 by ELISA.

Cytotoxicity and Coculture Assays. For CTL assays, splenocytes were plated at 1.5×10^7 cells/well in six-well dishes with 1.5×10^6 irradiated (20,000 rads) EG-7 cells or Ova (10 μg/ml) for 5 days. Then, splenocytes were harvested and plated against 1×10^4 ^{51}Cr-labeled target cells for 4 h in 96-well plates. Targets included EG-7, EL-4, and EL-4 that had been loaded (10 μg/ml for 2 h) with Ova peptide. For NK coculture and cytotoxicity assays, 2×10^6 DCs were cultured with 1×10^6 NK cells for 18 h in 600 μl before being plated in 96-well plates against 1×10^4 ^{51}Cr-labeled Yac-1 cells (ATCC) in a 4-h assay. Alternatively, freshly isolated NK cells were plated against Yac-1 cells. NK cells were isolated by incubating splenocytes with anti-NK (DX5) Microbeads (Miltenyi Biotech, Auburn, CA) before passing them through MACS high gradient LS separation columns (Miltenyi Biotech). Percentage of lysis was calculated as: [(experimental − spontaneous release) × 100]/(maximum release − spontaneous release). Spontaneous release was always <15% of maximum release.

RESULTS

Adenovirus Activates DC. To determine the effects of recombinant adenovirus on DCs, we infected bone marrow-derived DCs on day 8 of culture with AdGFP and examined them 2 days later by flow cytometry for transgene and surface marker expression. An MOI of 500 accomplished transgene expression in 40–50% of cells. Higher viral doses increased transgene expression but produced additional toxicity (Fig. 1*A*). Adenovirus infection up-regulated DC surface marker expression as reported previously (Refs. 10, 11; Fig. 1*B*). We found that adenovirus infection resulted in higher DC expression of MHC class II, CD54, CD80, and CD86 than CD40 ligation. However, TNF-α treatment induced greater surface expression of MHC class II and CD86 than adenoviral infection (Fig. 1*C*). Scanning electron microscopy showed that AdDC also had morphological features characteristic of mature DCs including ruffled cellular membranes and long dendritic processes (Fig. 1*D*).

DCs infected with adenovirus (MOI 500) produced 200 pg of IL-12 p70 per 10^6 DCs by 48 h after infection (Fig. 1*E*). Mock-infected DCs did not generate any detectable IL-12 in an ELISA (sensitivity = 62 pg/ml) or a bioassay (not shown; sensitivity = 10^{-3} pg/ml) using 2D6 cells, which proliferate specifically in response to IL-12. Although DCs infected with adenovirus secreted IL-12, we found that IL-12 protein alone, without adenoviral infection, did not cause DC maturation at concentrations as high as 100 ng/ml. Furthermore, IL-12 blockade did not inhibit or lessen the maturational effects of adenovirus infection (not shown). Finally, whereas infected DCs secreted IL-12, they did not produce detectable levels of IL-2, IL-4, IFN-γ, TNF-α, or GM-CSF by 48 h after infection as measured by ELISA.

Adenovirus Impairs Antigen Uptake and Chemotaxis. We reasoned that adenoviral infection of DCs likely hindered their ability to capture antigen and to migrate toward chemokines in keeping with the traditional concept of DC maturation. Macropinocytosis was assessed by capture of albumin, whereas endocytosis via the mannose receptor was tested by uptake of mannosylated albumin (20). In both cases, adenovirus inhibited antigen uptake in a dose-dependent manner although the effects were more striking with albumin (Fig. 2*A*). We evaluated DC chemotactic ability toward the Rantes chemokine. Infected DCs had impaired chemotaxis with a migrational index 55% lower than that of mock-infected DCs. Nevertheless, DCs infected with adenovirus still had a >2-fold higher migrational index than LPS-treated DCs (10 ng/ml; Fig. 2*B*).

Adenovirus Enhances T-Cell and NK Activation by DC. To determine the effect of adenoviral infection on DC ability to stimulate T cells, we first tested their allostimulatory capacity in an MLR. AdGFP-infected DCs induced markedly higher allogeneic proliferation than mock-infected DCs at all dilutions tested (Fig. 3*A*). We reasoned that the enhanced allostimulation might be related to the increased DC IL-12 production after adenoviral transduction. IL-12 has been shown previously to augment allostimulation at physiological doses (21). To test this, we repeated the MLR but added a blocking IL-12 antibody to wells containing AdDC. This blockade completely eliminated allostimulation by AdDC at T cell:DC ratios of 10:1 and 100:1 suggesting that the enhanced T-cell-stimulatory capacity of AdDC was attributable largely to IL-12 generation (data not shown). Next, we tested the effect of adenovirus infection on DC ability to induce antigen-specific T-cell stimulation. AdDC and mock-infected DCs were used alone or loaded with either Ova or ovalbumin before plating with Ova-restricted CD8$^+$ T-cell hybridoma cells. AdDC loaded with Ova or ovalbumin induced considerably higher IL-2 secretion compared with mock DCs loaded with antigen (Fig. 3, *B* and *C*). As observed with the MLR, we found that IL-12 blockade diminished T-cell activation of AdDC loaded with Ova by nearly 50% (Fig. 3*C*).

After showing that adenovirus infection alone greatly enhanced the T-cell-stimulatory capacity of DCs, we tested its effect on the ability of DCs to activate NK cells. Because IL-12 is a known stimulator of NK cells (22), we postulated that the IL-12 generated from AdDC would activate NK cells. Splenic NK cells cocultured with mock-infected DC failed to generate detectable IFN-γ and induced only 1% lysis of Yac-1 cells. Conversely, NK cells cocultured with AdDC produced 56 pg/ml of IFN-γ by 18 h and generated 12% Yac-1 lysis at a 50:1 E:T ratio (Fig. 4, *A* and *B*). IL-12 blockade reduced IFN-γ production by >50% indicating that IL-12 secretion by AdDC was in part responsible for their activation of NK cells.

Adenovirus Enhances DC Tumor Protection. Because DCs infected with adenovirus profoundly increased T-cell and NK cell stimulation *in vitro*, we hypothesized that they would have enhanced ability to generate antitumor immunity *in vivo*. To test their antitumor potential, we used a strategy of loading AdDC and the relevant controls with a model tumor antigen and subsequently using them to immunize mice against a tumor expressing that antigen. We immunized C57Bl/6 mice twice at weekly intervals with 5×10^5 AdDC.Ova (AdDC loaded with Ova peptide) and then challenged them 1 week later with a flank injection of EG-7 or an irrelevant tumor. Controls included mice immunized with saline, mock DC, DC.Ova, or AdDC not loaded with Ova peptide. All of the animals

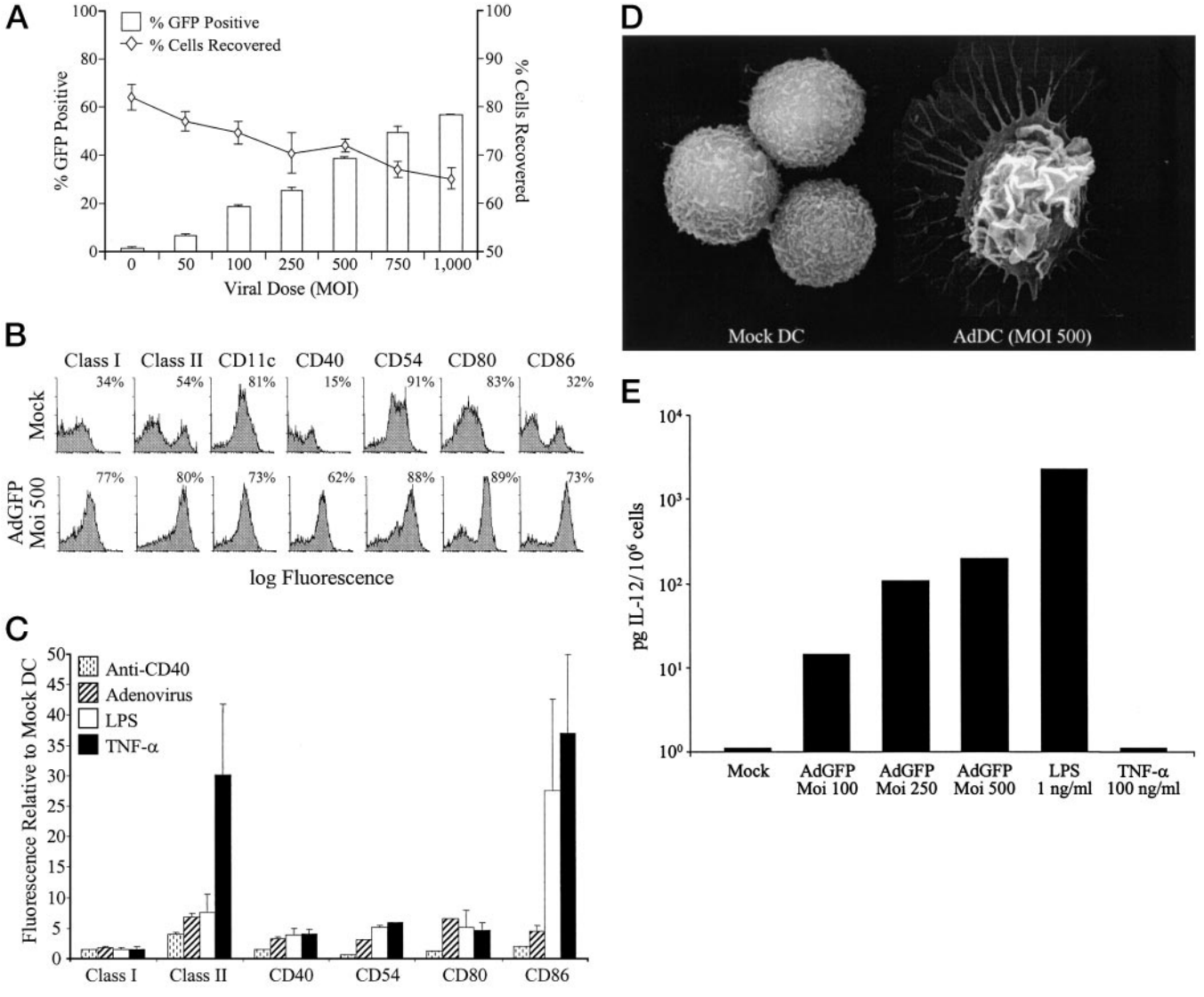

Fig. 1. Adenovirus activates DCs. DC (7×10^6 cells) from day 8 of bone marrow culture were infected in 7 ml of medium on a 100-mm plate with various doses of AdGFP. Approximately 18 h later, cells or supernatant were harvested for analysis. Data are representative of 4–10 experiments. *A*, increasing viral doses resulted in increased transgene (GFP) expression by FACS but lower cell viability by trypan blue exclusion. *B*, DCs infected with adenovirus had higher surface molecule expression by FACS than mock-infected DCs. *Top right corner* shows percentage of cells that are positive for each marker compared with isotype controls (not shown). *C*, comparison of DC maturation induced by overnight incubation with adenovirus, LPS (1 ng/ml), CD40 ligation (100 ng/ml of FGK45), and TNF-α (100 ng/ml). Fluorescence was normalized to that of mock DCs, which were assigned a value of 1. *D*, scanning electron micrographs (5; ×400) showed that mock-infected DCs (*left*) were rounded, whereas AdDC (*right*) had visible ruffles and extended dendritic processes. *E*, DCs infected with adenovirus secreted IL-12 in a dose response fashion but considerably less than even low dose LPS-treated DCs. In contrast, DCs matured by TNF-α did not secrete IL-12 even at doses of 100 ng/ml. The SE was <5%; *bars*, ±SE.

challenged with EL-4 (the same tumor as EG-7 not expressing Ova, $n = 4$/immunization group), B16 ($n = 4$/group), or Pan02 ($n = 5$/group) developed tumor regardless of their immunization. In addition, all of the mice ($n = 12$–14/group) immunized with saline, mock DC, or DC.Ova and then challenged with EG-7 also developed tumor. However, 69% of animals (9 of 13) immunized with AdDC.Ova were protected from developing EG-7 tumors. Surprisingly, even 31% of animals (4 of 13) immunized with AdDC alone were also protected. Conversely, DC.Ova conferred no protection (Fig. 5*A*). All of the protected animals in the AdDC or AdDC.Ova immunization groups remained free of tumor for the extent of their follow-up, which was as long as 180 days in many cases.

After establishing that immunization with DCs infected with adenovirus induces systemic immunity against tumor development, we wanted to determine whether immunological memory was generated. To this end, mice that did not develop tumor by at least 85 days after their initial inoculation with EG-7 were given a second inoculation of EG-7 cells in the opposite flank (Fig. 5*B*). We found that 56% (5 of 9) of animals in the AdDC.Ova immunization group and 67% (2 of 3) in the AdDC group were again protected from tumor development. The continued protection from EG-7 indicated that a memory immune response was elicited after immunization with infected DCs. Memory was Ova-specific as EL-4 tumors still developed in survivors of the initial tumor challenge ($n = 7$).

To determine the cellular mechanism underlying these observations, we performed depletion experiments before immunization. Depletion of $CD4^+$ T cells, $CD8^+$ T cells, or NK cells abrogated the full advantage of AdDC immunization (Fig. 5*C*). Whereas all of the nondepleted animals immunized with AdDC.Ova were protected in this experiment, tumor developed in 100% animals depleted of $CD8^+$

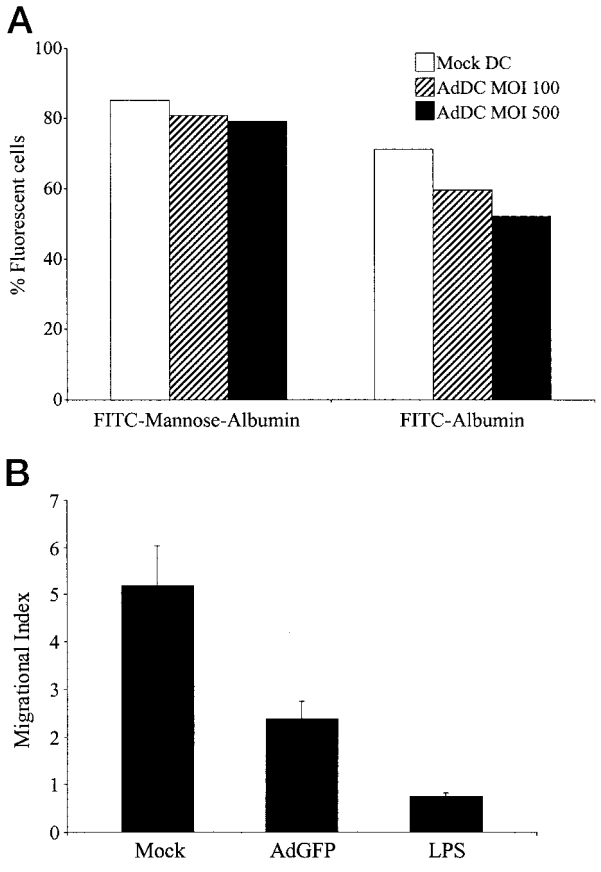

Fig. 2. DCs infected overnight with adenovirus impairs antigen capture and chemotaxis ability. *A*, DC capture of albumin (1 mg/ml) and mannosylated albumin (1 mg/ml) after 1 h culture were decreased in a dose-dependent fashion after overnight infection with AdAlkPhos. Data are representative of experiments repeated four times with similar results using 10,000 cells/group. $P < 0.0001$ by the χ^2 test. *B*, DC chemotaxis toward Rantes (500 ng/ml) over 2 h was decreased by >50% after infection. LPS had an even more pronounced adverse effect on Rantes directed migration. Data are representative of experiments repeated five times with similar results; *bars*, ±SE.

T cells, 60% of animals depleted of $CD4^+$ T cells alone, 100% of animals depleted of both $CD4^+$ and $CD8^+$ T cells, and 80% of animals depleted of NK cells. These data suggest that neither T cells alone nor NK cells alone were sufficient to confer complete antitumor immunity after immunization with infected DCs.

Because both NK and T cells were required for the full antitumor effects, we wanted to determine whether activated NK and T cells were actually generated *in vivo* after immunization with infected DCs. A single immunization of AdDC produced modest increases in splenic NK cell lytic activity against Yac-1 targets by 24 h (data not shown). To determine whether cytotoxic $CD8^+$ T cells were generated, we restimulated splenocytes from immunized animals *in vitro* with Ova and then plated them against ^{51}Cr-labeled EG-7 cells in a CTL assay (Fig. 6*A*). Similar results were seen when splenocytes were restimulated with EG-7 cells. Splenocytes from mice immunized with AdDC.Ova generated ~3-fold higher CTL activity than those from DC.Ova immunized mice. In concordance with the tumor challenge experiments, splenocytes from animals immunized with AdDC generated elevated lytic activity against EG-7 cells suggesting that AdDC immunization alone, without loading with a tumor antigen, generated cytotoxic T-cell activation. Animals depleted of NK cells using monoclonal antibodies did not have diminished CTL activity indicating that increased lysis was entirely $CD8^+$ T-cell mediated. The specificity of the CTL assay was demonstrated by the markedly lower (by ~50%) lysis against EL-4 targets, whereas lysis against EL-4 loaded with Ova was similar as EG-7 (not shown). As an additional control, splenocytes from mice immunized with AdDC.Ova or AdDC.βgal and restimulated *in vitro* with βgal failed to induce CTL against EG-7 targets or against EL-4 targets loaded with Ova indicating that effects were Ova-specific. Splenocytes from AdDC- or AdDC.Ova-immunized animals generated ~20 ng/ml of IFN-γ by 3 days after restimulation *in vitro* with Ova compared with just 7 ng/ml from animals immunized with mock-infected DCs (Fig. 6*B*). IFN-γ production was not diminished by prior NK cell depletion *in vivo* (data not shown). Besides IFN-γ overexpression, IL-4 was also differentially expressed after immunization with infected DCs (Fig. 6*B*).

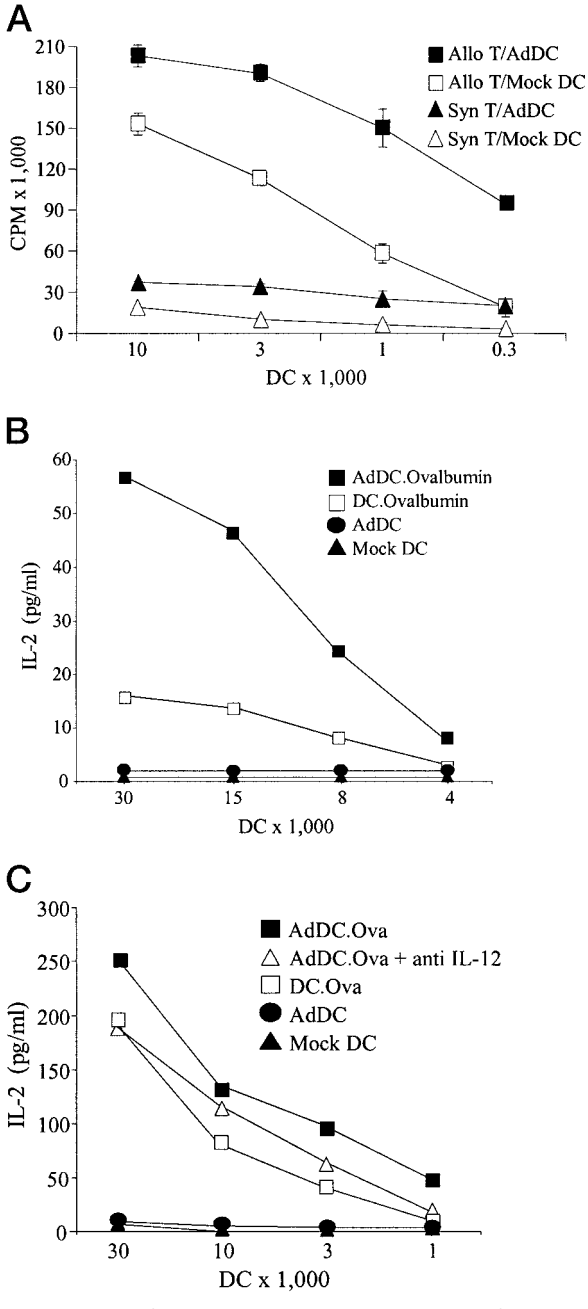

Fig. 3. Adenovirus infection increases allogeneic and antigen-specific T-cell stimulation. *A*, AdGFP-transduced DCs induced markedly higher proliferation of allogeneic (*Allo*) Balb/C T cells than mock DC, whereas syngeneic (*Syn*) T-cell proliferation was minimal. MLR experiments were repeated four times in triplicate with similar results *B* and *C*, AdDC caused higher IL-2 secretion from Ova-restricted T cells when loaded with (*B*) ovalbumin or (*C*) Ova peptide. The increased T-cell activation was reduced by an IL-12 blocking antibody. Antigen-restricted T-cell assays were repeated three times in triplicate with similar results. SE was <5% for the experiments shown; *bars*, ±SE.

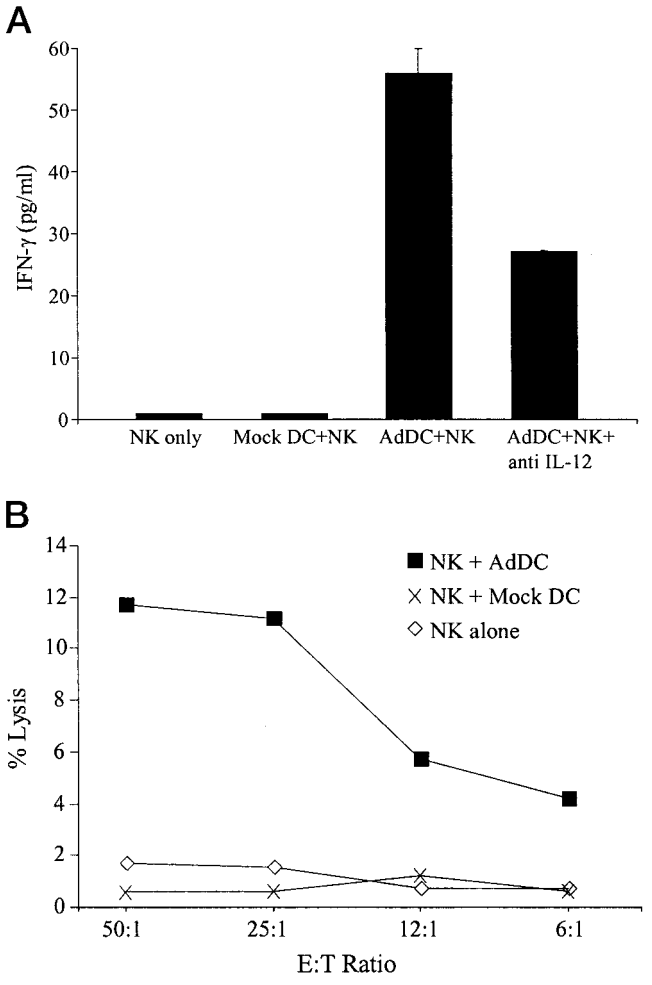

Fig. 4. Adenovirus infection enhances DC ability to activate NK cells *in vitro*. *A*, NK cells cocultured with AdDC for 18 h produced 56 pg/ml of IFN-γ compared with undetectable levels in control wells. This was reduced by IL-12 blockade. *B*, NK lytic activity against Yac-1 cells was also markedly higher after overnight coculture with AdDC. Coculture assays were repeated four times with similar results. SE was <5% for the cytotoxicity assay; *bars*, ±SE.

DISCUSSION

We have shown that an adenoviral vector alone, in the absence of a therapeutic transgene, induces profound changes in DC immune properties. DCs infected with adenovirus demonstrated substantial increases in T-cell stimulation compared with mock DCs in both allogeneic and antigen-specific models. Similarly, adenoviral infection resulted in enhanced DC activation of NK cells. The augmented T-cell and NK cell activation by AdDC was shown to be related to DC secretion of IL-12 after adenoviral infection. Furthermore, *in vivo* inoculation with AdDC conferred tumor protection via activation of T and NK cells, whereas mock-infected DCs provided no benefit. We have also shown that adenoviral infection slightly impairs DC antigen uptake and markedly reduces their chemotaxis. The knowledge that adenovirus alone induces significant changes in DC immune and antitumor function is critical to the evaluation of immunotherapy regimens using vector-based gene transfer to modulate immunity.

Adenoviral vectors are indeed powerful vehicles that can be used to overexpress selected proteins in DCs for therapeutic advantage (9, 23–25). However, a recent controversy has emerged as to the effects of the adenovirus vector itself on DC phenotype. Two initial reports using human DCs indicated that adenovirus alone had no, or only minor, effects on DC surface molecule expression (26, 27). Tillman *et al.* (27) reported that adenoviral infection only matured human DCs when the virus was targeted to CD40. However, without CD40 targeting, the adenovirus particle was incapable of inducing significant maturation. In contrast, subsequent reports using both murine (10, 11) and human (28) models indicated that adenovirus alone was fully capable of inducing DC maturation. We have consistently found that adenovirus markedly up-regulates several important DC surface molecules in murine DCs.

In the current report, DCs matured by adenovirus infection induced overwhelming allogeneic proliferation compared with mock-infected

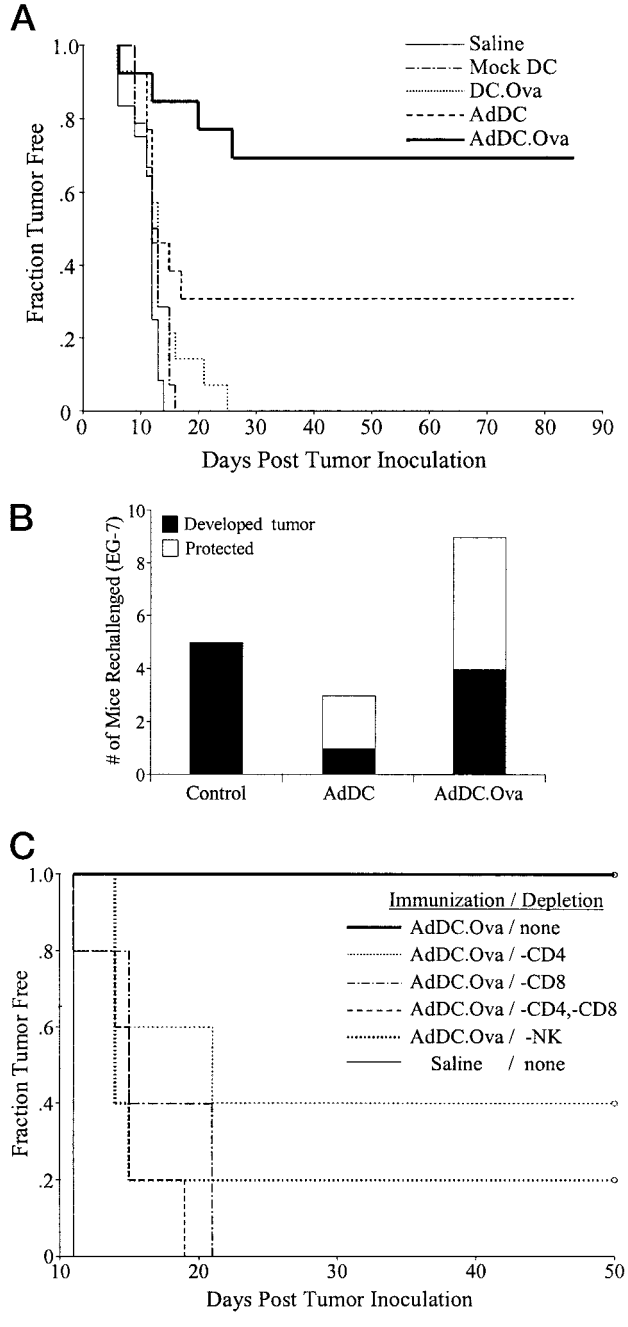

Fig. 5. Immunization with infected DCs confers systemic immunity against tumor development. *A*, survival of animals challenged with EG-7 shows that mice immunized with AdDC.Ova or AdDC were protected against EG-7 in 69% and 31% of cases, respectively (n = 12–14/group; $P < 0.0001$). *B*, 2 of 3 AdDC-immunized mice and 5 of 9 AdDC.Ova-immunized mice were immune to rechallenge with EG-7 at least 85 days after initial tumor inoculation. *C*, depletion of either $CD4^+$ or $CD8^+$ T cells or NK cells completely or partially abrogated immune protection against EG-7 conferred by immunization with infected DCs loaded with Ova (n = 5/group; $P < 0.01$).

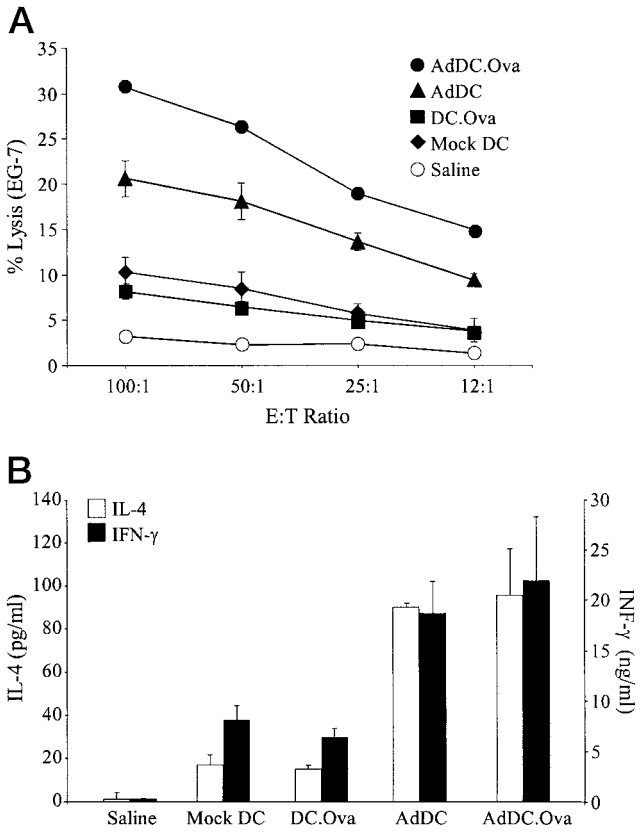

Fig. 6. Immunization with infected DCs generates cytotoxic T cells. *A*, immunization with AdDC or AdDC.Ova markedly increases CTL activity against EG-7 cells compared with immunization with mock-DC or DC.Ova. CTL experiments were done in triplicate and performed six times with similar results. SE was <5% for AdDC.Ova group in the experiment shown. *B*, both IFN-γ (ng/ml) and to a lesser extent IL-4 (pg/ml) were overexpressed in the CTL cultures of animals immunized with AdDC and AdDC.Ova; *bars*, ±SE.

DCs. Previous reports did not show such a striking increase in allostimulation by AdDC in both murine (11, 25) and human (26) models. Gong *et al.* (25) actually showed decreased alloproliferation induced by adenovirus infection at doses above an MOI of 200. However, the authors correlated the decreased alloproliferation at higher MOIs with increased DC death because of viral toxicity. In the present study, we used exclusively virally transduced live cells as determined by FACS sorting. Thus, neither the cytopathic effect of the virus nor admixture with uninfected DCs confounded the results. DCs matured by a variety of other methods including LPS, TNF-α, and CD40 ligation have also been reported to possess enhanced allostimulatory capacity (29–31). The precise mechanism for enhanced allostimulation by mature DCs is unknown. Whereas IL-12 blockade completely abrogated the enhanced proliferation of AdDC in our system (Fig. 3*B*), a previous report by Verhasselt *et al.* (31) indicated that IL-12 release by DCs matured with LPS was not responsible for their increased allostimulation. This implies that the effects of maturation on DC ability to activate effector cells may vary depending on the maturing stimulus. Our findings in Fig. 1, *C* and *E*, that different stimuli have varied effects on DC maturation and IL-12 secretion support this contention. We also showed that the augmented antigen-specific $CD8^+$ T-cell activation by AdDC was reduced somewhat by IL-12 blockade. Therefore, IL-12 plays at least a partial role in both allo- and antigen-specific T-cell stimulation. This was not surprising considering that IL-12 is a major T_h1 driving cytokine. It is important to note that whereas IL-12 blockade hindered the enhanced T-cell activation of AdDC, it did not prevent adenovirus-induced DC maturation. Thus, IL-12 is a critical byproduct of maturation with implications on AdDC function, but it is not the mediator of maturation itself.

Fernandez *et al.* (32) first reported that DCs can activate NK cells *in vitro* by contact, but only if the DCs were cultured in IL-4. DCs cultured only in GM-CSF, as in the present study, had no demonstrable effect on NK cells. However, we showed that DCs infected with adenovirus can differentially augment NK cell IFN-γ production and profoundly enhance their lytic activity even without IL-4 stimulation. NK activation by AdDC was again partially abrogated by IL-12 blockade. Our findings are consistent with the known capacity of IL-12 to activate NK cells and to stimulate them to secrete IFN-γ (22). The critical role of IL-12 in DC-NK interaction is also supported in a recent report of human peripheral blood mononuclear cell-derived DC activation of NK cells (33).

Previous studies have focused on using adenoviral vectors to transfer therapeutic transgenes to DCs to generate T-cell-mediated tumor protection (8, 9, 24). We have shown that immunization with infected DCs, without the benefit of a therapeutic transgene, conferred systemic protection against tumor development by generating cytotoxic T cells and NK cells. Our depletion experiments indicated that antitumor immunity required both T-cell and NK cell activation, whereas neither effector cell alone conferred sufficient protection in most animals (Fig. 5*C*). The inadequacy of the NK effect alone is additionally supported by the lack of protection against EL-4 after AdDC or AdDC.Ova immunization.

There are several lines of evidence suggesting that animals immunized with AdDC, without Ova loading, generated a T-cell response against EG-7. First, animals immunized with infected DCs but depleted of $CD8^+$ T cells ($n = 5$) all died indicating that activated NK cells alone were insufficient to protect against tumor and, therefore, could not account for the 31% protection from EG-7 seen in mice immunized with AdDC alone ($n = 13$). Secondly, 2 of 3 mice in the AdDC group exhibited a memory response to EG-7 (but not to EL-4) suggesting that a T-cell response was induced. Third, CTL activity was documented in the AdDC-treated animals. There was no protection against B16, Pan02, and EL-4 tumors. The precise mechanism for T-cell activation after AdDC immunization (without Ova loading) is uncertain and is possibly related to their release of IL-12. It seems unlikely that a substantial number of injected DCs survived until tumor challenge. Our findings raise the potential for AdDC to initiate autoimmunity through the capture and presentation of self-antigens, although we did not observe this in the long-term survivors.

Mock-infected DCs, either alone or pulsed with Ova, were unable to generate cytotoxic T or NK cells, or induce a sufficient antitumor immune response presumably because of their lack of maturation. DCs require activation before exhibiting their full immunostimulatory properties (1), and incubation with Ova alone does not result in DC maturation (data not shown). Conversely, AdDC.Ova immunized mice received the full benefit of both antigen loading as well as DC maturation, and were, therefore, fully capable of activating NK cells and Ova-specific T cells. The result was immunity against tumor development in 78% of animals in the combined experiments ($n = 18$).

Because adenovirus alone profoundly alters DC ability to capture antigen, migrate, stimulate effector cells *in vitro* and *in vivo*, and induce protective immunity against tumors, these experiments form a vital baseline for current and future investigations, and protocols using adenoviral-mediated gene transfer to DC. Moreover, our findings raise critical issues regarding the effects of the vector alone and may have important implications to other systems using vector-based gene transfer to modulate immunity.

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REFERENCES

1. Banchereau, J., and Steinman, R. M. Dendritic cells and the control of immunity. Nature (Lond.), *392:* 245–252, 1998.
2. Jenne, L., Arrighi, J. F., Jonuleit, H., Saurat, J. H., and Hauser, C. Dendritic cells containing apoptotic melanoma cells prime human CD8+ T cells for efficient tumor cell lysis. Cancer Res., *60:* 4446–4452, 2000.
3. Kikuchi, T., Miyazawa, N., Moore, M. A., and Crystal, R. G. Tumor regression induced by intratumor administration of adenovirus vector expressing CD40 ligand and naive dendritic cells. Cancer Res., *60:* 6391–6395, 2000.
4. Nouri-Shirazi, M., Banchereau, J., Bell, D., Burkeholder, S., Kraus, E. T., Davoust, J., and Palucka, K. A. Dendritic cells capture killed tumor cells and present their antigens to elicit tumor-specific immune responses. J. Immunol., *165:* 3797–3803, 2000.
5. Kugler, A., Stuhler, G., Walden, P., Zoller, G., Zobywalski, A., Brossart, P., Trefzer, U., Ullrich, S., Muller, C. A., Becker, V., Gross, A. J., Hemmerlein, B., Kanz, L., Muller, G. A., and Ringert, R. H. Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids. Nat. Med., *6:* 332–336, 2000.
6. Nestle, F. O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G., and Schadendorf, D. Vaccination of melanoma patients with pep. Nat. Med., *4:* 328–332, 1998.
7. Di Nicola, M., Siena, S., Bregni, M., Longoni, P., Magni, M., Milanesi, M., Matteucci, P., Mortarini, R., Anichini, A., Parmiani, G., Drexler, I., Erfle, V., Sutter, G., and Gianni, A. M. Gene transfer into human dendritic antigen-presenting cells by vaccinia virus and adenovirus vectors. Cancer Gene Ther., *5:* 350–356, 1998.
8. Furumoto, K., Arii, S., Yamasaki, S., Mizumoto, M., Mori, A., Inoue, N., Isobe, N., and Imamura, M. Spleen-derived dendritic cells engineered to enhance interleukin-12 production elicit therapeutic antitumor immune responses. Int. J. Cancer, *87:* 665–672, 2000.
9. Melero, I., Duarte, M., Ruiz, J., Sangro, B., Galofre, J., Mazzolini, G., Bustos, M., Qian, C., and Prieto, J. Intratumoral injection of bone-marrow derived dendritic cells engineered to produce interleukin-12 induces complete regression of established murine transplantable colon adenocarcinomas. Gene Ther., *6:* 1779–1784, 1999.
10. Hirschowitz, E. A., Weaver, J. D., Hidalgo, G. E., and Doherty, D. E. Murine dendritic cells infected with adenovirus vectors show signs of activation. Gene Ther., *7:* 1112–1120, 2000.
11. Morelli, A. E., Larregina, A. T., Ganster, R. W., Zahorchak, A. F., Plowey, J. M., Takayama, T., Logar, A. J., Robbins, P. D., Falo, L. D., and Thomson, A. W. Recombinant adenovirus induces maturation of dendritic cells via an NF-κB-dependent pathway. J. Virol., *74:* 9617–9628, 2000.
12. Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R. M. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J. Exp. Med., *176:* 1693–1702, 1992.
13. Lutz, M. B., Kukutsch, N., Ogilvie, A. L., Rossner, S., Koch, F., Romani, N., and Schuler, G. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J. Immunol. Methods, *223:* 77–92, 1999.
14. DeMatteo, R. P., Chu, G., Ahn, M., Chang, E., Burke, C., Raper, S. E., Barker, C. F., and Markmann, J. F. Immunologic barriers to hepatic adenoviral gene therapy for transplantation. Transplantation (Baltimore), *63:* 315–319, 1997.
15. Rolink, A., Melchers, F., and Andersson, J. The SCID but not the RAG-2 gene product is required for S μ-S epsilon heavy chain class switching. Immunity, *5:* 319–330, 1996.
16. Maruo, S., Ahn, H. J., Yu, W. G., Tomura, M., Wysocka, M., Yamamoto, N., Kobayashi, M., Hamaoka, T., Trinchieri, G., and Fuijiwara, H. Establishment of an IL-12-responsive T cell clone: its characterization and utilization in the quantitation of IL-12 activity. J. Leukoc. Biol., *61:* 346–352, 1997.
17. Wilkinson, V. L., Warrier, R. R., Truitt, T. P., Nunes, P., Gately, M. K., and Presky, D. H. Characterization of anti-mouse IL-12 monoclonal antibodies and measurement of mouse IL-12 by ELISA. J. Immunol. Methods, *189:* 15–24, 1996.
18. Capsoni, F., Minonzio, F., Ongari, A. M., and Zanussi, C. A new simplified single-filter assay for '*in vitro*' evaluation of chemotaxis of 51Cr-labeled polymorphonuclear leukocytes. J. Immunol. Methods, *120:* 125–131, 1989.
19. Carbone, F. R., Sterry, S. J., Butler, J., Rodda, S., and Moore, M. W. T cell receptor α-chain pairing determines the specificity of residue 262 within the Kb-restricted, ovalbumin257–264 determinant. Int. Immunol., *4:* 861–867, 1992.
20. Sallusto, F., Cella, M., Danieli, C., and Lanzavecchia, A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J. Exp. Med., *182:* 389–400, 1995.
21. Kelleher, P., and Knight, S. C. IL-12 increases CD80 expression and the stimulatory capacity of bone marrow-derived dendritic cells. Int. Immunol., *10:* 749–755, 1998.
22. Chan, S. H., Kobayashi, M., Santoli, D., Perussia, B., and Trinchieri, G. Mechanisms of IFN-γ induction by natural killer cell stimulatory factor (NKSF/IL-12). Role of transcription and mRNA stability in the synergistic interaction between NKSF and IL-2. J. Immunol., *148:* 92–98, 1992.
23. Kikuchi, T., Worgall, S., Singh, R., Moore, M. A., and Crystal, R. G. Dendritic cells genetically modified to express CD40 ligand and pulsed with antigen can initiate antigen-specific humoral immunity independent of CD4+ T cells. Nat. Med., *6:* 1154–1159, 2000.
24. Song, W., Kong, H. L., Carpenter, H., Torii, H., Granstein, R., Rafii, S., Moore, M. A., and Crystal, R. G. Dendritic cells genetically modified with an adenovirus vector encoding the cDNA for a model antigen induce protective and therapeutic antitumor immunity. J. Exp. Med., *186:* 1247–1256, 1997.
25. Gong, J., Chen, L., Chen, D., Kashiwaba, M., Manome, Y., Tanaka, T., and Kufe, D. Induction of antigen-specific antitumor immunity with adenovirus-transduced dendritic cells. Gene Ther., *4:* 1023–1028, 1997.
26. Zhong, L., Granelli-Piperno, A., Choi, Y., and Steinman, R. M. Recombinant adenovirus is an efficient and non-perturbing genetic vector for human dendritic cells. Eur. J. Immunol., *29:* 964–972, 1999.
27. Tillman, B. W., de Gruijl, T. D., Luykx-de Bakker, S. A., Scheper, R. J., Pinedo, H. M., Curiel, T. J., Gerritsen, W. R., and Curiel, D. T. Maturation of dendritic cells accompanies high-efficiency gene transfer by a CD40-targeted adenoviral vector. J. Immunol., *162:* 6378–6383, 1999.
28. Rea, D., Schagen, F. H., Hoeben, R. C., Mehtali, M., Havenga, M. J., Toes, R. E., Melief, C. J., and Offringa, R. Adenoviruses activate human dendritic cells without polarization toward a T-helper type 1-inducing subset. J. Virol., *73:* 10245–10253, 1999.
29. Brunner, C., Seiderer, J., Schlamp, A., Bidlingmaier, M., Eigler, A., Haimerl, W., Lehr, H. A., Krieg, A. M., Hartmann, G., and Endres, S. Enhanced dendritic cell maturation by TNF-α or cytidine-phosphate-guanosine DNA drives T cell activation *in vitro* and therapeutic anti-tumor immune responses *in vivo*. J. Immunol., *165:* 6278–6286, 2000.
30. Canque, B., Camus, S., Yagello, M., and Gluckman, J. C. IL-4 and CD40 ligation affect differently the differentiation, maturation, and function of human CD34+ cell-derived CD1a+. J. Leukoc. Biol., *64:* 235–244, 1998.
31. Verhasselt, V., Buelens, C., Willems, F., De Groote, D., Haeffner-Cavaillon, N., and Goldman, M. Bacterial lipopolysaccharide stimulates the production of cytokines and the expression of costimulatory molecules by human peripheral blood dendritic cells: evidence for a soluble CD14-dependent pathway. J. Immunol., *158:* 2919–2925, 1997.
32. Fernandez, N. C., Lozier, A., Flament, C., Ricciardi-Castagnoli, P., Bellet, D., Suter, M., Perricaudet, M., Tursz, T., Maraskovsky, E., and Zitvogel, L. Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses *in vivo*. Nat. Med, *5:* 405–411, 1999.
33. Amakata, Y., Fujiyama, Y., Andoh, A., Hodohara, K., and Bamba, T. Mechanism of NK cell activation induced by coculture with dendritic cells derived from peripheral blood monocytes. Clin. Exp. Immunol., *124:* 214–222, 2001.