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Immunosuppressive effects of interleukin-12 coexpression in melanoma antigen gene–modified dendritic cell vaccines

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Genetic immunotherapy with tumor antigen gene–modified dendritic cells (DC) generates robust immunity, although antitumor protection is not complete in all models. Previous experience in a model in which C57BL/6 mice immunized with DC transduced with adenoviral vectors expressing MART-1 demonstrated a 20–40% complete protection to a tumor challenge with B16 melanoma cells. Tumors that did develop in immunized mice had slower growth kinetics compared to tumors implanted in naïve mice. In the present study, we wished to determine if the supraphysiological production of the Th1-skewing cytokine interleukin-12 (IL-12) could enhance immune activation and antitumor protection in this model. In a series of experiments immunizing mice with DC cotransduced with MART-1 and IL-12, antitumor protection and antigen-specific splenocyte cytotoxicity and interferon γ production inversely correlated with the amount of IL-12 produced by DC. This adverse effect of IL-12 could not be explained by a direct cytotoxic effect of natural killer cells directed towards DC, nor the production of nitric oxide leading to down-regulation of the immune response — the two mechanisms previously recognized to explain immune-suppressive effects of IL-12–based vaccine therapy. In conclusion, in this animal model, IL-12 production by gene-modified DC leads to a cytokine-induced dose-dependent inhibition of antigen-specific antitumor protection.
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Genetic immunization with tumor antigen–transduced dendritic cell (DC) generates antigen-specific antitumor responses.[1–5] The endogenous expression of tumor antigen genes to provide antigenic epitopes for both MHC classes I and II–restricted expression,[6,7] together with high level of costimulatory and adhesion molecule expression by DC,[8] makes gene-modified DC one of the most powerful means of generating antitumor immunity.[9,10] Using human MART-1 as a tumor antigen, we have previously demonstrated that adenovirally transduced DC generate protective immunity to the murine melanoma B16,[11–13] which expresses the murine counterpart of MART-1 with a 69% sequence homology.[14] Mice immunized with AdVMART-1/DC develop antigen-specific MHC class I–restricted CTLs with a type 1 immune phenotype.[1,7,11,12] This response is dependent on the immunizing DC and not on crosspriming,[11] and requires $CD4^+$ T-cell help mediated by the crosslinking of CD40 receptor on the gene-modified DC.[13] The superior protective effect of viral vector–mediated gene-modified DC has been reproduced in multiple other models, some of them with unpreceded antitumor protective or treatment effects.[9,10] However, our experience has shown that only a minority of AdVMART-1/DC-immunized mice are completely protected when challenged with a single cell suspension of B16 melanoma cells obtained from tumors growing *in vivo* in syngeneic mice, thereby avoiding the confounding immunological effect of media and fetal calf serum–derived epitopes.[1] As there is room for improved protection, we hypothesized that an enhanced Th1-biasing cytokine production may improve antitumor protection in this stringent murine model of immunotherapy for melanoma.

Interleukin-12 (IL-12) is a heterodimeric cytokine initially termed natural killer (NK) cell stimulating factor because of its ability to stimulate NK cells,[15] and cytotoxic lymphocyte maturation factor by another group because of its ability to synergize with IL-2 for the generation of CTL.[16] Among the multiple effects attributed to IL-12,[17,18] an important biological function is the induction of

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interferon γ (IFN-γ) production, which biases the immune system towards a cytotoxic response with a Th1 phenotype. In fact, IL-12 appears to be the key cytokine skewing an immune response to a Th1 response and it can promote a switch from an established Th2 to a Th1 response.[17,18] These functions have led to the study of IL-12 as an ideal adjuvant for cancer immunotherapy in preclinical and clinical studies.[17–22] In the present studies, we attempted to enhance the protective immunity generated by melanoma antigen gene–modified DC by vaccinating mice with DC cotransduced with IL-12 and the melanoma antigen MART-1. Our results indicate that IL-12 coexpression by DC not only failed to enhance antitumor immunity in this model, but it led to a dose-dependent inhibition of protection to B16 melanoma.

Materials and methods

Mice and cell lines

C57BL/6 and B6.129S2-Cd8a^{tm1Mak} (CD8 KO) mice (both H-2^b) were purchased from the Jackson Laboratory (Bar Harbor, ME) and were bred and kept in the pathogen-defined Animal Facility of the Division of Experimental Radiation Oncology at the University of California at Los Angeles (UCLA). Female mice 5–8 weeks old were used for all studies. Mice were handled in accordance with the UCLA animal care policy. B16, a murine melanoma, and EL4, a murine lymphoma, were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and maintained *in vitro* in DMEM (GIBCO, Rockville, MD) with 10% FCS (Gemini Products, Calabasas, CA), 1% (vol/vol) penicillin, streptomycin, and fungizone (Gemini Products) (complete media). Yac-1, a Moloney leukemia virus–induced murine lymphoma, was also obtained from ATCC (TIB 160) and maintained *in vitro* in RPMI (GIBCO) complete media. EL4 (MART-1) was developed by transfection of the parental line with a plasmid (pRcCMVMART-1) carrying the MART-1 cDNA and *neo* resistance gene as previously described.[11] Stably transfected cells were maintained *in vitro* under constant G418 selection (0.5 mg/mL; GIBCO).

Recombinant adenoviruses

AdVMART-1 is an E1-deleted replication-deficient adenoviral vector based on human type 5 adenovirus. The construction and characterization of this vector have been described previously.[1,23] The adenovirus murine IL-12.1 (AdVIL-12) vector, a generous gift from Dr Frank Graham (McMaster University, Ontario, Canada), has also been previously described.[24,25] This vector contains the p35 and p40 subunit cDNA of murine IL-12 in the early regions 1 (E1) and 3 (E3), respectively, of adenovirus type 5. In both vectors, the transgenes are driven by the human cytomegalovirus (CMV) early promoter/enhancer. The biologic and functional activity of AdVIL-12 was confirmed by quantitation of IL-12 production by a p70 IL-12 ELISA (Pharmingen, San Diego, CA), and by the detection of a stimulation of IFN-γ production *in vivo* after intratumoral administration.[25]

Preparation of DC and adenoviral transduction

DC were differentiated from murine bone marrow progenitor cells by *in vitro* culture in GM-CSF and IL-4 as described by Inaba et al[26] with minor modifications.[1] *In vitro* cultured DC were transduced in 15mL conical tubes (Costar, Acton, MA) in a final volume of 1 mL of RPMI with 2% FCS, to which the virus stock was added at a multiplicity of infection (MOI) of 100 viral plaque-forming units (pfu) per DC (or MOI of 200 if the transduction was performed in a final volume of 2 mL). Transduction was carried out for 2 hours at 37°C, after which time the DC were washed extensively and resuspended in 0.2 mL of PBS per animal for injection into mice. Cell counts were determined using a hemocytometer with viability assessed by trypan blue exclusion. In all cases, viability exceeded 95%. Transduction of murine DC with the replication-defective adenoviral vector AdVMART-1 (AdVMART-1/DC) between MOI of 1 and 100 results in MART-1 expression that persists for at least 5 days.[1] The level of IL-12 cytokine production by DC transduced with cytokine-expressing adenoviral vectors was determined by p70 IL-12 ELISA (Pharmingen).

Animal studies

Mice were immunized on days 1 and 8 with $1–5\times10^5$ DC/mouse administered subcutaneously in the right flank, and challenged in the left flank 10–14 days after the last immunization with B16 (7×10^4 to 1×10^5 cells/animal). Cells used for tumor challenge were obtained from single cell suspensions of progressively growing tumors in syngeneic mice to avoid the confounding effects of the presentation of media- and serum-derived epitopes.[1] Cell suspensions were washed extensively and injected into mice in a final volume of 0.2 mL of PBS per animal. Typically, six mice were immunized per group, five used for *in vivo* challenge with B16 and one for immunological assays. Occasionally, a total of five mice per group were used.

In vivo *depletion of CD8$^+$ T and NK cell subsets*

In vivo monoclonal antibody ablation of CD8$^+$ (clone 2.43, ATCC TIB 210) or NK (clone PK136, ATCC TIB 160; purified asialo-GM1, Cedarlane, Honrnby, Ontario, Canada) cell subsets was performed by intraperitoneal injection on days 5, 3, and 1 before tumor inoculation, and every 7 days thereafter (0.5 mg antibody/mouse/injection). Antibody suspensions were purified from clone 2.45 and PK136 hybridoma supernatants by passage through Protein G columns according to the manufacturer's instructions (Pierce, Rockford, IL). Eluted immunoglobulins were dialyzed against PBS and stored at 4°C in 1 mg/mL suspensions. CD8$^+$ T-cell depletion was confirmed by flow cytometric analysis of splenocytes from depleted mice on the day of tumor challenge. NK depletion was confirmed by NK cell activity microcytotoxicity assays using the NK-sensitive Yac-1 cell line as target cells (Yac-1 assay).

Cytotoxicity assays

For *in vitro* short-term microcytotoxicity assays, splenocytes from one mouse from each treatment group were harvested 14 days after the last immunization, depleted of red blood

cells by hypotonic lysis, restimulated *in vitro* with irradiated EL4 (MART-1) at a 25:1 ratio for 96 hours in the presence of 10 U/mL IL-2, and assayed in a standard 4-hour chromium release assay against chromated B16 cells.[7,11,12] For Yac-1 assay of NK activity, splenocytes from naïve, NK cell–depleted and CD8 knockout mice were harvested and cultured in 10,000 IU/mL recombinant IL-2 for 16–18 hours after red cell depletion. These cells were tested for their ability to lyse chromated Yac-1 cells or transduced DC in a 16-hour chromium release assay. For each different target, samples were tested against their own maximum and spontaneous release.

Cytokine profile by ELISPOT

For IFN-γ and IL-4 ELISPOT assays, red blood cell–depleted splenocytes, restimulated *in vitro* for 48 hours at the same conditions described above for cytotoxicity assays, were added in duplicate 3-fold dilutions to 96-well mixed cellulose plates (Multiscreen filtration system; Millipore, Bedford, MA) precoated with anti–IFN-γ or anti–IL-4 antibody (Pharmingen) as previously described.[7,11,12] After 24-hour incubation at 37°C, plates were washed and incubated at 4°C with secondary biotinylated antibody. On the next day, spot-forming colonies were developed by the addition of horseradish peroxidase avidin D (Vector Laboratories, Burlingame, CA) followed by color reaction using 3-amino-9-ethyl-carbazole (AEC; Sigma, St. Louis, MO). Spots were counted under a dissecting microscope.

Coculture and determination of nitric oxide (NO)

Transduced and untransduced DC were resuspended at 5×10^5 DC/mL in DMEM complete media, and 1 mL was added to wells of 24-well plates containing the same amount of media with 2×10^6 red blood cell–depleted splenocytes obtained from naïve mice. Each sample was plated into six replicate wells. For studies aimed at determining the NO production after immunization with AdVMART-1+IL-12/DC *in vivo*, spleens were harvested 24 hours after immunization and the adherent fraction containing macrophages was cultured for 48 hours *in vitro*. Splenocytes stimulated with 100 μg/mL LPS and 100 U/mL IFN-γ were plated as a positive control. At 48 hours, supernatants were harvested and assayed for the presence of NO by chemiluminescence as previously described.[27] This method is based on the detection of total NO_x (NO+nitrite [NO_2^-]+nitrate [NO_3^-]) by chemiluminescence after reduction of NO_x to NO by acidic vanadium (III), thereby providing an accurate assessment of the chemically unstable NO in oxygen-containing solutions.

Statistical analysis

Results of *in vivo* studies are presented as the mean and standard error of the mean (SEM) of tumor volumes in each treatment group. Mice completely protected from a tumor challenge are presented separate from mice that did develop tumors to allow correct assessment of the rate of tumor growth.[1] If a treatment group is divided into mice with and without tumors in a tumor volume plot, the number of mice with (or without) tumor from that group over the total number of mice in the group is shown in parenthesis. Significance is calculated using the *t* test or the Rank Sum Test.

Results

Cotransduction of DC with adenoviral vectors expressing MART-1 and murine IL-12 decreases antitumor protection

DC cotransduced with AdVMART-1 and AdVIL-12 produce IL-12 in a viral dose-dependent fashion. GM-CSF/IL-4 differentiated DC transduced with AdVMART-1 produce 0.17 ng of IL-12/10^6DC/24 hours as analyzed by ELISA. When cotransduced with AdVIL-12 at an MOI of 1:10, IL-12 production increased to 3.2 ng/10^6DC/24 hours, and at an MOI of 1:100, IL-12 production further increased to 19 ng/10^6DC/24 hours. In three replicate studies, C57BL/6 mice were immunized with DC cotransduced with AdVMART-1 and AdVIL-12 (AdVMART-

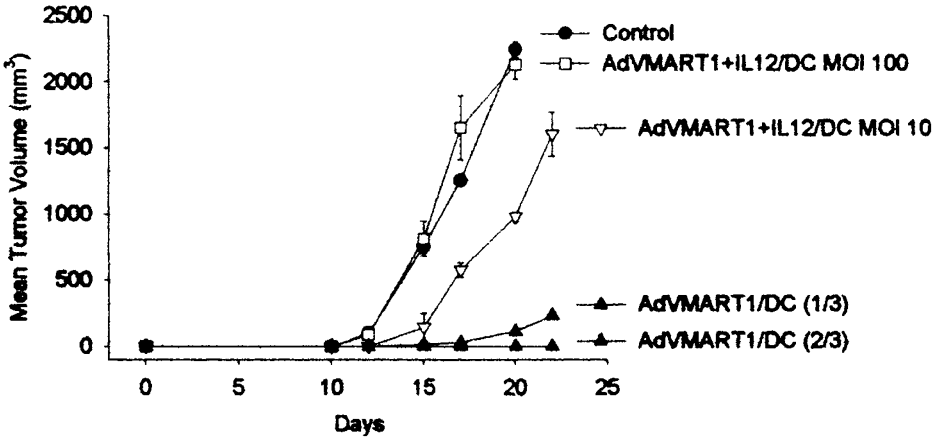

Figure 1 Antitumor protection after immunization with AdVMART-1+IL-12/DC. Groups of mice were immunized with DC transduced with AdVMART-1 at an MOI of 100, with or without further transduction with AdVIL-12 at an MOI of 10 or 100. After two weekly subcutaneous immunizations, mice were challenged with a single cell suspension of B16 cells obtained from tumors growing in syngeneic mice. Mice immunized with AdVMART-1/DC were significantly protected from a tumor challenge with B16 when compared to AdVMART-1+IL12/DC at an MOI of 10 (P=.003) or MOI of 100 (P=.0008).

1+IL-12/DC). Compared with mice immunized with AdVMART-1-transduced DC, coexpression of IL-12 impaired the rejection of a tumor challenge with B16 melanoma cells (Fig 1). In fact, AdVIL-12 transduction at an MOI of 1:100 completely abrogated the protective effect of AdVMART-1/DC vaccination, suggesting a cytokine dose-dependent effect. These studies were repeated in another well-established murine genetic immunization tumor model using AFP as tumor antigen,[28] again demonstrating an adverse impact of IL-12 cotransduction on antitumor immunity (data not shown).

Decreased antigen-specific Th1 cytokine production and in vitro *cytotoxicity by IL-12 coexpression in DC*

Mice immunized with AdVMART-1/DC generate MART-1–specific splenic cells with a type 1 cytokine phenotype able to lyse B16 melanoma cells in microcytotoxicity assays.[11] Spleens from mice immunized with AdVMART-1/DC cotransduced with AdVIL-12 were restimulated *in vitro* with MART-1–expressing cells in the presence of low concentrations of IL-2. At 48 hours, splenocytes were analyzed for the presence of IFN-γ–producing cells by ELISPOT. The number of splenocytes that produced of this type 1 cytokine after MART-1 *ex vivo* restimulation decreased with increasing AdVIL-12 MOI (Fig 2A). After 96 hours of *ex vivo* restimulation, the ability to lyse chromated B16 melanoma cells was assayed in microcytotoxicity assays. The lysis observed with AdVMART-1/DC vaccination was completely abrogated in splenocytes from mice immunized with AdVMART-1+IL-12/DC (Fig 2B). Therefore, these immunological assays confirm the findings from *in vivo* studies.

IL-12–producing DC vaccines are not eliminated by NK cells

IL-12 can activate NK cells,[15] and we considered the possibility that IL-12–transduced DC resulted in a NK-mediated destruction of the vaccines. NK-depleted mice immunized with AdVMART-1/DC have an intermediate level of antitumor protection (Fig 3A), suggesting that NK cells play some role in the antitumor response mediated by gene-modified DC, a finding in accord with other reports.[29] However, NK depletion had no impact on IL-12–mediated suppression of antitumor immunity (Fig 3A). Splenocytes from NK-depleted and nondepleted mice were harvested 2 weeks after DC vaccinations and assessed for MART-1–induced IFN-γ production by ELISPOT. A comparable decrease in the frequency of IFN-γ–producing cells was noted both in NK-depleted and nondepleted mice (Fig 3B). Also, DC were not good targets for NK lysis because NK cells generated in high concentrations of IL-2 could not lyse AdVMART-1+IL-12/DC, AdVMART-1/DC, or untransduced DC in cytotoxicity assays, even though they had high lytic activity against NK-sensitive Yac-1 targets (Fig 3C).

Systemic immunosuppression by IL-12–transduced DC

The immunosuppressive effect of IL-12 in this model appeared to have a systemic rather than a paracrine mechanism. Mice were immunized with MART-1 and IL-12–cotransduced DC on one flank, and AdVMART-1/DC in the contralateral flank. These mice had comparable degrees of depressed immunity as those receiving only unilateral AdVMART-1+IL-12/DC (Fig 3D), with an equivalent decrease in MART-1–induced IFN-γ–producing cells in these two groups. These data argue in favor of a global immunosuppressive effect rather than a paracrine inhibitory effect of IL-12 on the locally injected DC vaccine.

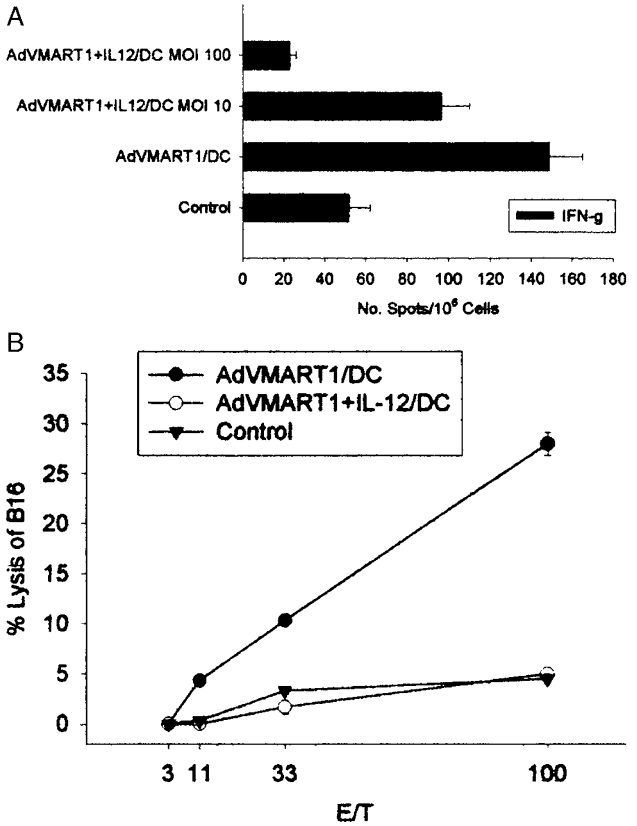

Figure 2 *In vitro* analysis of antigen-specific immunity. **A:** IFN-γ ELISPOT. Groups of mice were immunized with DC transduced with AdVMART-1 at an MOI of 100 and AdVIL-12 at an MOI of 10 or 100. Two weeks after the last immunization, splenocytes were harvested and restimulated *in vitro* with irradiated EL4 (MART-1) for 48 hours, and the number of IFN-γ–producing cells was assayed by ELISPOT. **B:** Cytotoxicity to B16. Splenocytes of mice generated as in **(A)** were restimulated for 96 hours, and then assayed in a standard 4-hour chromium release for the ability to lyse chromated B16 cells.

Lack of induction of NO production by MART-1 and IL-12–transduced DC

IL-12 has been shown to have an immune-suppressive effect under certain conditions, which has been attributed to an IFN-γ–induced activation of iNOS in macrophages, leading to NO production and suppression of T-cell responses.[30] Therefore, we determined whether AdVMART-1+IL-12/DC stimulated IFN-γ and NO production when cocultured *ex vivo* with splenocytes. For these studies, DC were differentiated in GM-CSF/IL-4, transduced with AdVMART-1 and AdVIL-12, and cocultured with syngeneic splenocytes derived from naïve mice at a

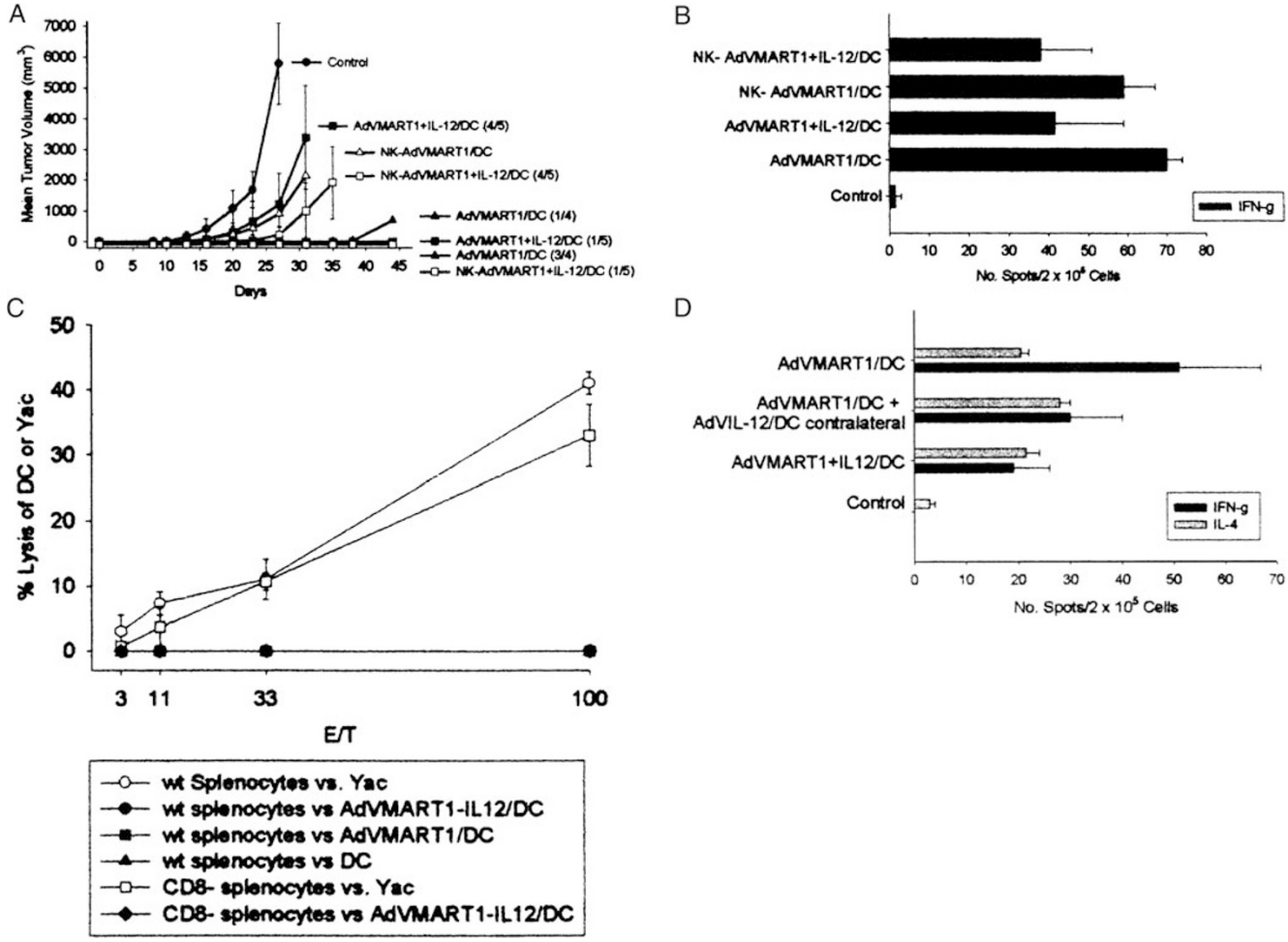

Figure 3 Effect of NK cells on AdVMART-1+IL-12/DC vaccines. **A:** Protection of B16 in mice depleted of NK cells. Mice were immunized with DC transduced with AdVMART-1 and AdVIL-12 and then groups of mice were depleted of NK cells. Mice were challenged with a single cell suspension of B16 cells. Significant differences can be noted between mice immunized with AdVMART-1/DC and AdVMART-1+IL-12/DC (P=.03), and AdVMART-1+IL-12/DC with NK depletion (P=.02), and marginally significant differences when compared to AdVMART-1/DC with NK depletion (P=.06). **B:** *In vitro* analysis of the effect of NK depletion after AdVMART-1+IL-12/DC immunization. Splenocytes from similarly immunized mice were restimulated with EL4 (MART-1) and the frequency of IFN-γ–producing cells was analyzed by ELISPOT. **C:** Lytic activity to DC. Splenocytes from naïve and CD8 knockout mice were activated overnight in culture containing high concentrations of IL-2 to stimulate NK activity. The lytic activity was assayed against the NK-sensitive Yac-1 cell line as a positive control, or to chromated DC with or without AdVMART-1 or AdVIL-12 transduction in a 16-hour chromium release assay. **D:** Systemic *versus* local effect of AdVMART-1+IL-12/DC immunization. Groups of mice were immunized with AdVMART-1/DC, AdVMART-1+IL-12/DC, or AdVMART-1+IL-12/DC with the contralateral injection of AdVMART-1/DC to determine the local *versus* systemic immune-suppressive effect of DC coexpressing MART-1 and IL-12. Splenocytes were harvested 2 weeks later and assayed for IFN-γ or IL-4 production in an ELISPOT assay.

1:4 ratio for 48 hours. After that time, cells were harvested and the frequency of IFN-γ and IL-4–producing cells was assessed by ELISPOT. As shown in Figure 4a, splenocytes from nonvaccinated mice alone had a baseline of IL-4–producing cells, but no IFN-γ production. Untransduced DC and DC transduced with AdVMART-1 cocultured with naïve splenocytes had similar populations of IL-4–producing cells, with a small population of IFN-γ–producing cells. On the contrary, splenocytes cocultured with DC transduced with AdVMART-1 and AdVIL-12 had a 10-fold increase in IFN-γ–producing cells, which confirms that AdVIL-12 produces a biologically active heterodimeric IL-12 protein. The supernatant of replicate wells was harvested and used to determine the NO content using the chemiluminescence method (Fig 4B). As a positive control, splenocytes were stimulated with LPS and IFN-γ for 48 hours, which induced a doubling of total NO production compared to cultures with splenocytes alone. However, AdVMART-1+IL-12/DC did not induce NO production on splenocytes as compared to AdVMART-1/DC, untransduced DC, or splenocytes alone.

The IL-12–induced and IFN-γ– and NO-mediated immunosuppressive effect has been noted *in vivo*.[30–33] To determine if AdVMART-1+IL-12/DC induced IFN-γ and NO production *in vivo* after systemic administration, mice were immunized with DC transduced with AdVIL-12 at different MOI and administered intravenously, a route that preferentially targets the spleen in prior experience.[7] After 24 hours, spleens were harvested. The adherent fraction containing macrophages was used to determine the

production of NO, and nonadherent cells were used to determine the frequency of nonantigen-specific production of IFN-γ and IL-4. As seen in Figure 5A, the number of IFN-γ–producing splenocytes by ELISPOT assay was very low in all the conditions, whereas the number of IL-4–producing cells was significantly higher. Adherent splenocytes were cultured for 48 hours and the supernatants assayed for NO contents (Fig 5B). As positive control, adherent splenocytes from a naïve mouse were cultured in LPS and IFN-γ, which led to a 3-fold increase in NO production. Samples from mice immunized with AdVMART-1+IL-12/DC had no increase in NO content compared to naïve mice or mice immunized with AdVMART-1/DC. In conclusion, our data do not support an enhanced NO production stimulated by IL-12–expressing DC as an explanation for the down-regulation of MART-1–specific responses by IL-12 in this model.

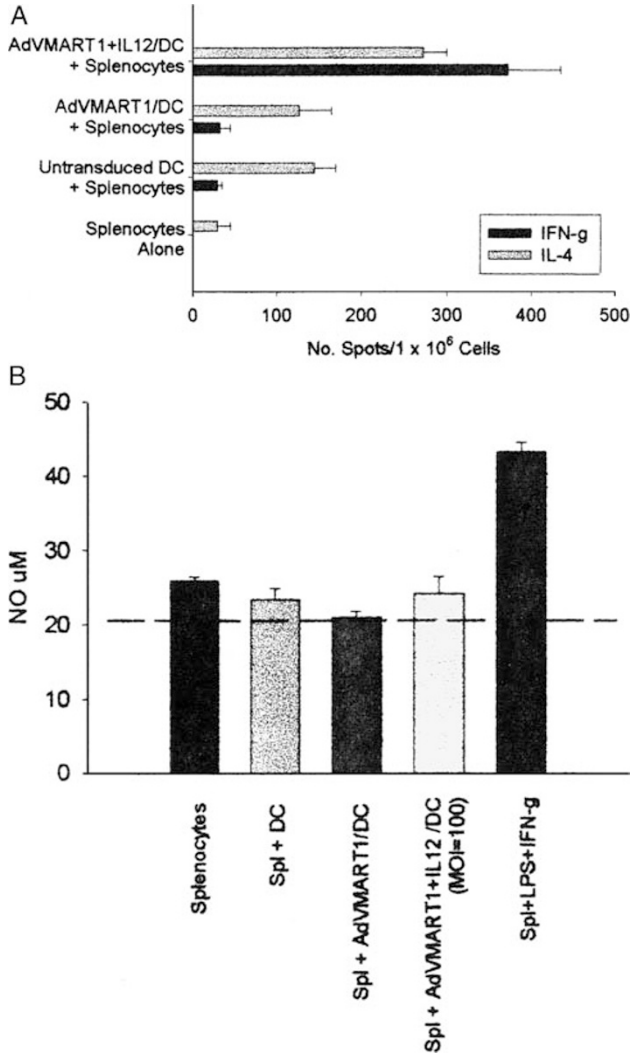

Figure 4 Production of IFN-γ and NO by naïve splenocytes cocultured *in vitro* with AdVMART-1+IL-12/DC. DC were transduced with AdVMART-1 and AdVIL-12 and cocultured with naïve splenocytes. At 48 hours, cells were harvested and analyzed for the presence of IFN-γ–producing cells by ELISPOT **(A)**, or for NO production by chemiluminescence **(B)**.

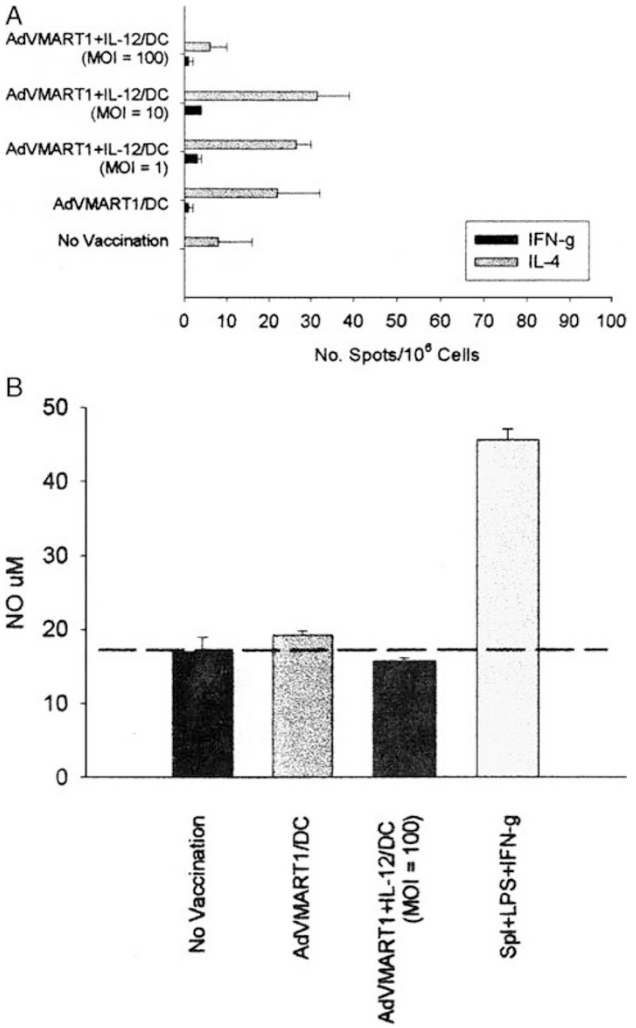

Figure 5 Production of IFN-γ and NO after immunization of mice with AdVMART-1+IL-12/DC. Mice were immunized with transduced and untransduced DC and spleens were harvested 24 hours later. Nonadherent splenocytes containing lymphocytes were assayed for the presence of IFN-γ–producing cells by ELISPOT **(A)**, and adherent cells containing macrophages were analyzed for NO production by chemiluminescence **(B)**.

Discussion

Protective antitumor immune responses generated by DC-based immunization have been linked to the ability to induce polarized Th1 responses.[34,35] Immature DC produce very low levels of IL-12. Maturation of DC by CD40 ligation, LPS, or viral infection leads to enhanced IL-12 production, which is thought to be the major factor promoting a Th1 response. This Th1 skewing results in the generation of cytotoxic T-cell activity as opposed to a humoral response.[36] Koch et al[37] have shown that baseline production of IL-12 is below the limit of detection in GM-CSF/IL-4 *in vitro* differentiated murine DC, which increases to 0.4 ng/10^6/

72 hours after CD40 engagement and 2 ng/10^6/72 hours after activation with a T-cell hybridoma. Hochrein et al[38] have analyzed the levels of IL-12 by different subsets of murine DC, and have reported that $CD8^+$ DC, supposedly of lymphoid origin, are the highest producers of IL-12 after CD40 engagement, in the order of 0.4 ng/mL, compared to less than 0.1 ng/mL when $CD8^-$ DC are similarly engaged with CD40.

Therefore, one strategy may be to skew the DC phenotype by providing the Th1-skewing cytokine IL-12 to favor stronger protective immunity. In preclinical models of infectious diseases and cancer, the administration of recombinant IL-12[39–42] or the transduction of DC with IL-12–expressing viral vectors[43–49] enhanced T-cell immunity. Zitvogel et al[43] first described the adjuvant effect of IL-12 transfection on the antitumor immunity generated by peptide-pulsed DC, which has been corroborated in other models.[46,47] Furthermore, intratumoral injection of IL-12–transduced DC also demonstrated an enhancement of the ability of DC to generate responses to tumors *in vivo*.[48–50] In these models, paracrine production of IL-12 by DC at levels of 2–40 ng/10^6/24 hours enhanced antitumor immunity. In contrast with these reports, we have shown a dose-dependent inhibition of antitumor immunity when immunizing with IL-12 and MART-1 genetically engineered DC vaccines. This is a systemic and not a paracrine effect and is mediated neither by NK cells nor NO production.

The adenovirus vector expressing IL-12 used in these studies is biologically active because direct injection of this vector into tumor-bearing mice leads to tumor rejection,[25] and it induces IFN-γ production by splenocytes (Figure 4A). IL-12 is physiologically produced by antigen-presenting cells (APC) with a Th1-type cytokine expression pattern, and its targets are T lymphocytes, NK, and NKT cells.[19,51–55] Once IL-12 engages its receptor, intracellular signaling through the signal transducer and activator of transcription 4 (STAT-4) leads to a promotion of a Th1 phenotype through the production of IFN-γ at the same time it inhibits Th2 cytokine production.[56] Furthermore, paracrine production of IL-12 at tumor sites inhibits blood vessel formation, and this antiangiogenic effect is a likely explanation for nonimmune IL-12–mediated responses in other murine models.[25,57]

IL-12 was originally described as a potent stimulator of NK cell activity.[15] The interaction between NK and DC is currently not clearly understood. Data suggest that DC have the ability to directly activate NK cells thorough an unrecognized receptor–ligand pair and the production of stimulatory cytokines like IL-12, providing a bridge between the innate and adaptive immune systems.[58] NK cells have also been shown to have a DC lytic effect. This may be surprising at first because DC are among the highest expressers of surface MHC molecules,[8] which are recognized by the Ly49 family of NK receptors in mice, and the killer inhibitory receptors (KIR) in humans, leading to a powerful inhibition of the NK-mediated lytic activity.[59] However, a high expression of surface B7 costimulatory molecules, as is also the case on DC, has been shown to override the MHC-induced inhibition of NK lytic activity.[60,61] Therefore, DC vaccines producing high concentrations of IL-12 may become targets for NK lysis before they are recognized by MART-1–specific T cells. When we explored this possibility, we failed to detect an NK-mediated lytic effect on the IL-12–transduced DC.

In several models, an IL-12 dose-dependent inhibitory effect has been reported. Recombinant IL-12 administered at low doses (1–100 ng/day) potentiated antiviral and antitumor responses, but at higher doses (100–1000 ng/day), it inhibited them.[62–64] A similar bimodal effect of high doses of recombinant IL-12 as an adjuvant for cancer vaccines in human subjects has been reported, with inhibition of tumor antigen–specific responses at doses greater than 300 ng/kg.[20,21] In the murine models, the effects of IL-12–induced immune suppression were linked to an enhanced IFN-γ production, which in turn activated iNOS in macrophages, leading a NO-induced suppression of T-cell responses.[30–33,65] However, when we tested this possibility in our model, we detected an enhanced IFN-γ production as an early response to IL-12, but no induction of iNOS. A direct way to asses the role of iNOS in this model would have been the use of iNOS knockout mice, which was not done in the current studies. Due to the multiple effects of high doses of IL-12 on other cytokines, an alternative explanation may include the induction of cytokines other than IFN-γ, leading to altered STAT signaling pathways that inhibit iNOS induction, which leads to down-regulation of NO production as proposed in other models.[66]

Alternative explanations for these observations are a cytokine shift and an altered epitope processing stimulated by IL-12–transduced DC. Immunization with IL-12 and MART-1 double-transduced DC resulted in lower levels of IFN-γ–producing splenocytes compared to transduction with MART-1 alone, but equivalent IL-4 production. Therefore, this shift from a Th1 to a Th2 response with IL-12 transduction may explain our observation, although it has been clearly established that IL-12 is the key cytokine in the induction of Th1 responses.[36] It is also possible that IL-12 may have altered proteasome processing and epitope presentation of MART-1–derived peptides by the DC. The IFN-inducible proteasome subunits stimulated by IL-12 may have altered MART-1 epitope processing, leading to a different set of peptides presented by these DC. These two possibilities were not studied in the current experiments.

The widely variable effects and results of IL-12–mediated therapy in animal models and human clinical trials suggest that this cytokine has a significant dependence on the model, concentration, and delivery method.[17,18,55,62,63,65,67,68] The timing, dose, and location of IL-12 administration have been shown to have immunostimulatory,[22,44,67,68] immune-suppressive,[30,31,62–65] and antiangiogenic[25,57] effects in different models. Therefore, its effect as a vaccine adjuvant should be carefully studied and used with caution, as it may in fact inhibit instead of potentiating vaccines. In conclusion, we have reported for the first time a dose-dependent adverse effect of genetic modification of DC to express IL-12 on the ability to immunize with tumor antigen gene–modified DC.

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