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Immune Deviation and Fas-mediated Deletion Limit Antitumor Activity after Multiple Dendritic Cell Vaccinations in Mice[1]

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ABSTRACT

Genetic immunization with a single injection of dendritic cells (DCs) expressing a model melanoma antigen generates antigen-specific, MHC-restricted, protective immune responses. After initiating the immune response, additional vaccinations may increase the protection or conversely downregulate the immune response. Groups of mice were vaccinated several times with DCs transduced with the *MART-1* gene, and the antitumor protection was compared with that of mice receiving a single vaccination. C3H mice had poorer protection from a syngeneic MART-1-expressing tumor challenge with multiple vaccinations. This was accompanied by lower levels of splenic CTL effectors and a shift from a type 1 to a type 2 cytokine profile. On the contrary, multiple vaccinations in C57BL/6 mice generated greater *in vivo* antitumor protection with no decrease in splenic CTLs and no cytokine shift. Antiadenoviral humoral or cellular immune responses did not seem to contribute to these effects. When studies were performed in Fas receptor-negative C3H.^{lpr} mice, the adverse effect of multiple vaccinations disappeared, and there was no cytokine shift pattern. In conclusion, C3H mice but not C57BL/6 mice receiving multiple vaccinations with DCs expressing the MART-1 tumor antigen show decreased protection associated with deviation from a type 1 to a type 2 cytokine response attributable to a Fas-receptor mediated clearance of antigen-specific IFN-γ-producing cells.

INTRODUCTION

DCs[3] are a small subset of leukocytes with a pivotal role in initiating and controlling immune responses. The development of efficient methods for *in vitro* generation of large quantities of DCs, both in mice (1) and in humans (2), has enabled testing of these cells in immunotherapy strategies. Tumor antigen presentation by DCs has been shown to efficiently initiate antitumor responses in a variety of animal models (3–6) and in human clinical trials (7, 8). However, DCs are also critical in regulating the immune response after it has been initiated and have been implicated in the induction of tolerance to organ allografts (9) and in tumor-induced tolerance (10, 11).

There are several theoretical advantages for the use of gene-modified DCs expressing tumor antigens. When recombinant adenoviral vectors are used to transduce DCs, high transgene levels are expressed, and peptide epitopes are appropriately endogenously processed and presented in a MHC class I and II-restricted fashion (12, 13). In extensive studies using MART-1 as a model tumor antigen, we have demonstrated the ability of adenovirally transduced DCs to generate robust antigen-specific protective responses in mice (5, 6, 14) and in humans (12, 13). However, an unexpected observation arose: C3H mice receiving multiple vaccinations had poorer protection compared with mice receiving a single vaccination (6). Furthermore, preliminary observations from human clinical trials using DC-based immunizations to induce tumor antigen-specific responses suggest a similar finding in humans (15, 16). In two trials in which DCs are pulsed with HLA-matched immunodominant peptides derived from the melanoma antigen-3 (15), tyrosinase, or gp100 tumor antigens (16), detection of serum antigen-specific cells peaked after a single DC administration and decreased thereafter to background levels. In the present studies, we extend on our previous experiments in mouse models to further examine this phenomenon and investigate its mechanism. Our data demonstrate different effects of multiple DC vaccinations in two strains of mice and suggest a role of type 1/type 2 cytokines and Fas/Fas ligand in mediating the decreased protection with multiple immunizations.

MATERIALS AND METHODS

Mice and Cell Lines. Female C3H mice (H-2^k), 5–8 weeks of age, were bred and kept in the Animal Facility of the Division of Experimental Radiation Oncology at the University of California Los Angeles. C57BL/6 mice (H-2^b; 5–8 weeks of age) and C3H.MRL-Fas^{lpr} (C3H.^{lpr}) mice (H-2^k; 3–4 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were handled in accordance with the University of California at Los Angeles animal care policy. The following cell lines were used: NFSA (a nonimmunogenic fibrosarcoma) and SVEC (an endothelial cell line) in studies in C3H and C3H.^{lpr} mice; and B16 (a melanoma), EL4 (a lymphoma), and 3LL (a lung carcinoma) in studies in C57BL/6 mice. All cell lines were obtained from the American Type Culture Collection (Rockville, MD). NFSA, SVEC, and 3LL were maintained *in vitro* in RPMI 1640 (Life Technologies, Gaithersburg, MD) with 10% FCS (Gemini Products, Calabasas, CA) and 1% (v/v) penicillin, streptomycin, and fungizone (Gemini Products; complete media). B16 and EL4 were maintained *in vitro* in DMEM (Life Technologies) complete media. NFSA(MART-1), SVEC(MART-1), EL4(MART-1), and 3LL(MART-1) were developed by transfection of the parental line with a plasmid (pRcCMVMART-1) carrying the MART-1 cDNA and *neo* resistance gene, as described previously (5, 6). Stably transfected cells were maintained *in vitro* under constant G418 selection (0.5 mg/ml; Life Technologies) in RPMI 1640.

Recombinant Adenoviruses. The replication-deficient adenoviral vectors used in this study are E1-deleted vectors based on human type 5 adenoviruses. The construction of these vectors has been described previously. AdVMART1 contains the 400-bp human MART-1 cDNA driven by the cytomegalovirus early enhancer/promoter, and AdVRR5 does not contain any gene insert (5, 12).

Preparation of DC and Adenoviral Transduction. DCs were differentiated from murine bone marrow progenitor cells by *in vitro* culture in granulocyte/macrophage-colony stimulating factor and IL-4 as described by Inaba *et al.* (1) with modifications (5, 6, 14). *In vitro* cultured DCs were transduced in 15-ml conical tubes (Costar, Acton, MA) in a final volume of 1 ml of RPMI 1640 with 2% FCS, to which the virus stock was added at a MOI of 100 viral pfu per each DC. Transduction was carried out for 2 h at 37°C, after which time the DCs 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 DCs with the replication-defective

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[3] The abbreviations used are: DC, dendritic cell; IL, interleukin; ELISPOT, enzyme-linked immunospot; MOI, multiplicity of infection; pfu, plaque-forming unit(s); APC, antigen-presenting cell; HA, hemagglutinin; AICD, activation-induced cell death.

adenoviral vector AdVMART1 (AdVMART1/DC, DCs transduced with AdVMART1) between MOI of 1 and 100 results in MART-1 expression that persists for at least 5 days (5, 6).

Animal Studies. Mice were immunized with 5×10^5 DCs/mouse administered i.v. through a lateral tail vein or s.c. in the right flank and were challenged on the left flank 7–14 days after the last immunization with NFSA(MART-1) (1×10^6/animal) in C3H and C3H.^{lpr} mice or with B16 (1×10^5/animal), 3LL(MART-1) (5×10^5/animal), or EL4(MART-1) (5×10^5/animal) in C57BL/6 mice. Cells used for tumor challenge were obtained from single-cell suspensions of progressively growing tumors in syngeneic mice to avoid the confounding effects of presentation of medium- and serum-derived epitopes (5, 6). Cell suspensions were washed extensively and injected into mice in a final volume of 0.2 ml of PBS/animal.

Adenovirus Neutralization Assay. Serum from C3H mice was harvested 10 days after the last immunization with AdVMART1/DC (5×10^5/injection) administered i.v. or with AdVMART1 (1×10^8 pfu/injection) administered i.p. Serum samples were heat inactivated at 56°C for 30 min and stored at −70°C. Serial dilutions of serum samples were incubated for 1 h at 37°C with 1×10^4 pfu of AdVLacZ in a final volume of 100 μl. Samples were used to transduce HeLa or 293 cells. Samples used to infect HeLa cells were diluted 1:2 and added to wells with 1×10^4 HeLa cells (0.5 MOI) and allowed to infect for 2 h. Wells were washed twice with PBS and incubated for 24 h in complete media. Cells were stained with β-galactosidase, and blue staining cells were counted using a microscope. Samples used to infect permissive 293 cells were diluted 1:200 after the serum neutralization and were titrated by further serial dilution, absorbed for 1 h, and overlaid with medium containing agarose. Cultures were incubated for 7 days and read for plaque formation.

Cytotoxicity Assays. For *in vitro* microcytotoxicity assays, splenocytes were harvested 7–14 days after the last immunization, depleted of RBCs by hypotonic lysis, restimulated *in vitro* with irradiated SVEC(MART-1) in C3H and C3H.^{lpr} mice or with EL4(MART-1) in C57BL/6 mice at a 25:1 ratio for 96 h in the presence of 10 units/ml of IL-2, and assayed in a standard 4-h chromium release test. To block perforin-mediated target cell lysis, effector cells were added in the presence of a final concentration of 3 mM EGTA/2 mM $MgCl_2$. For each different target, samples were tested against their own maximum and spontaneous release.

Cytokine Profile by ELISPOT. ELISPOT assays were performed by adding RBC-depleted splenocytes, restimulated *in vitro* with the same conditions as above for cytotoxicity assays but for 48 h, 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, San Diego, CA), as described previously (6, 14). After 24-h incubation at 37°C, plates were 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 (Sigma Chemical Co., St. Louis, MO). Spots were counted under a dissecting microscope.

Statistical Analysis. Differences in tumor development were assessed using the χ^2 or the Fisher exact test. Results of *in vivo* studies are presented as the mean and SE of tumor volumes in each treatment group. Mice completely protected from a tumor challenge are presented separately from mice that did develop tumors to allow correct assessment of the rate of tumor growth (5, 6, 14). Significance is calculated using the *t* test (or the Rank Sum Test in case of failing the Kolmogorov-Smirnov test for Normality). Each study included at least five mice per group and has been repeated at least twice.

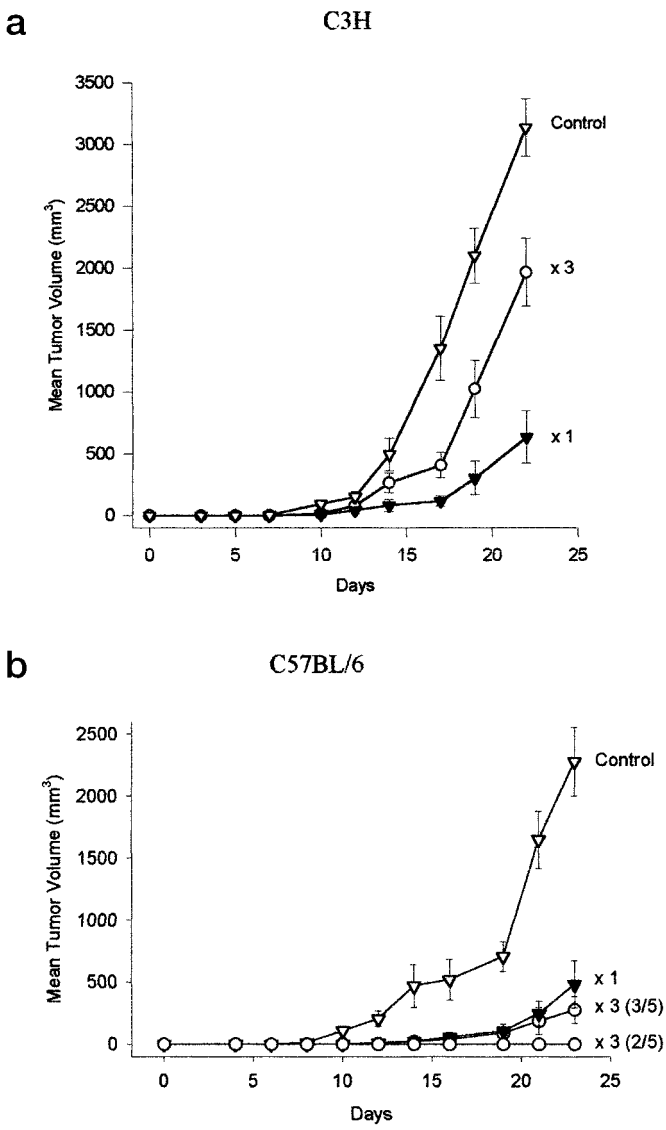

Fig. 1. Tumor development and growth in immunocompetent mice immunized different times with AdVMART1/DC. *a*, C3H mice were immunized once (▼) or three times (○) i.v. with 5×10^5 AdVMART1/DC per animal and were challenged 10 days later with NFSA(MART-1) cells obtained from tumors grown in syngeneic mice (five mice/group). Time is counted from the date of tumor challenge. Significant differences were noted on final tumor volumes between mice immunized once and those immunized three times ($P = 0.01$). Data are representative of seven of eight replicate studies. *b*, C57BL/6 mice were immunized once (▼) or three times (○) s.c. with 5×10^5 AdVMART1/DC per animal and were challenged 10 days later with 3LL(MART-1) cells obtained from tumors grown in syngeneic mice. Differences in final tumor volumes are not significant, although more mice in the group immunized three times as compared with the group immunized once were completely protected from tumor challenge (two of five *versus* zero of five, respectively; *P* not significant). Similar results were obtained in a replicate study using 3LL(MART1) and in two studies using B16 tumor challenge (data not shown). *Bars*, SD.

RESULTS

Multiple Immunizations with MART-1-engineered DCs Exhibit Mouse Strain-specific Differences in Elicited MART-1 Immunity. Both C3H mice (H-2^k) and C57BL/6 (H-2^b) mice immunized with a single AdVMART1-transduced DC injection exhibited protection from a MART-1-expressing tumor challenge (5, 6, 14). In an effort to improve the degree of protection, we examined the effect of multiple AdVMART1/DC immunizations in these two mouse strains. Multiple weekly AdVMART1/DC immunizations elicited progressively poorer protection to tumor challenge in C3H mice but improved protection in C57BL/6 mice (Fig. 1). This is a highly reproducible phenomenon. Results were consistent in seven of eight C3H mouse experiments (total mice, 145) in which the groups of mice receiving a single vaccination had smaller tumors than mice receiving three or five vaccinations. In C57BL/6 experiments (total mice, 60), the same pattern of improved protection with multiple vaccinations was observed in two of two studies challenged with 3LL(MART-1) and in two of two studies challenged with the murine MART-1-positive melanoma B16.

Effects of the Number of AdVMART1/DC Immunizations in the Generation of MHC-restricted, MART-1-specific CTLs and IFN-γ-producing T Cells. Multiple AdVMART1/DC immunizations generate lower CTL responses in C3H mice and slightly better

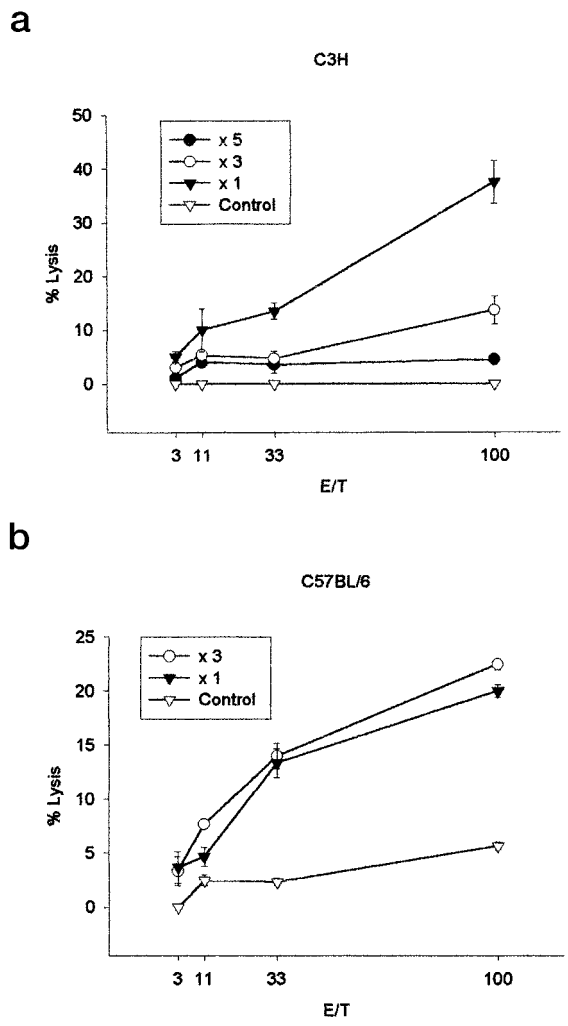

Fig. 2. *In vitro* lytic activity of restimulated splenocytes from mice immunized different numbers of times. Mice were vaccinated with 5×10^5 AdVMART1/DC per animal. Ten days later, splenocytes were harvested and restimulated with irradiated SVEC(MART-1) (*a*) or EL4(MART-1) (*b*) cells (25:1 ratio) plus 10 units/ml of IL-2. CTL assays were performed on the 5th day. Each figure represents a separate study, and each study has been repeated at least twice with similar results. *a*, lytic activity in splenocytes derived from C3H mice immunized five times (●), three times (○), or once (▼) or not immunized (▽). *B*, lytic activity in C57BL/6 mice immunized once (▼) or three times (○) or not immunized (▽). *Bars*, SD.

(certainly no worse) responses in C57BL/6 mice (Fig. 2). We also observed a reproducible pattern of MART-1-specific type 1 (IFN-γ) and type 2 (IL-4) cytokine patterns in the splenocytes used for CTL assays. C3H mice had progressive type 1 to type 2 shift in cytokine production with additional immunizations. Fewer IFN-γ-producing cells but greater numbers of IL-4-producing cells were found in the multiply immunized but poorly protected C3H strain (Fig. 3*a*). An increment in both IFN-γ and IL-4 was observed in the C57BL/6 strain (Fig. 3*b*). This effect is not dependent on the route of immunization because a similar cytokine pattern was observed in mice receiving multiple s.c. (Fig. 3*c*) or i.v. (Fig. 3*d*) vaccinations. In both mouse strains, type 1 and type 2 cytokine production was MART-1 specific. Immunization with DCs transduced with a non-MART-1-expressing type-specific adenoviral vector (AdVLacZ; Fig. 3*a*) and *in vitro* restimulation with non-MART-1-expressing cells (Fig. 3*b*) stimulated less cytokine-producing cells in ELISPOT assays.

Effects of Antiviral Responses on the Generation of MART-1 Immunity. Responses to epitopes derived from the adenoviral backbone of AdVMART1 may have an adverse effect in mice receiving multiple vaccinations. To determine whether antiadenoviral neutralizing antibodies were generated after multiple AdVMART1/DC vaccinations in C3H mice, sera from mice immunized once or three times with AdVMART1/DC were assayed and compared with sera from naive mice or from mice immunized three times with AdVMART1 injected i.p. (1×10^8 pfu/injection). Sera from mice immunized with AdVMART1 administered systemically contained high titers of adenovirus neutralizing antibodies, whereas antiadenoviral neutralizing antibodies were low in sera obtained from mice immunized once with AdVMART1/DC (Fig. 4*a* and data not shown). However, neutralizing antibodies to the adenoviral backbone increased in C3H mice immunized three times with AdVMART1/DC. To determine the role of antiadenoviral cellular responses after multiple adenovirus/DC vaccinations, C3H mice were immunized several times with DCs transduced with an adenoviral vector not expressing the *MART-1* gene (AdVRR5) and received a final vaccination with AdVMART1/DC. As shown in Fig. 4, *b* and *c*, mice exposed two or four times to adenovirally transduced DCs were still able to generate a MART-1 response, as demonstrated by the ability to protect from an *in vivo* tumor challenge and to have MART-1-specific, IFN-γ-producing cells. Therefore, humoral and cellular responses to the adenoviral vector do not explain the adverse effect of multiple AdVMART1/DC vaccinations in C3H mice.

Reversal of the Adverse Effect of Repeated AdVMART1/DC Vaccinations in Fas Receptor-negative Mice. Cells from mice with a mutation in the Fas receptor are not susceptible to Fas/Fas ligand-mediated lysis, a mechanism which might be involved in limiting immune responses to strong antigenic stimulation (18). To determine the role of Fas/Fas ligand in this model, young Fas receptor-negative C3H.^{lpr} mice were immunized several times with AdVMART1/DC. Ten to 14 days after the last immunization, mice were either challenged with NFSA(MART-1) or their were splenocytes harvested for ELISPOT and microcytotoxicity assays. In contrast to Fas receptor-normal C3H mice, tumor appearance and tumor growth rate in C3H.^{lpr} mice were not adversely influenced by repeated AdVMART1/DC vaccinations in two separate studies (Fig. 5*a*). Also, *in vitro* cytotoxicity was not decreased in restimulated splenocytes from C3H.^{lpr} mice receiving repeated vaccinations (Fig. 5*b*). This pattern was not attributable to increased Fas ligand-dependent target cell lysis, because blocking calcium-dependent pathway (presumably perforin-mediated) using Mg^{2+}-EGTA completely abrogated target cell lysis (data not shown). In five ELISPOT assays, C3H.^{lpr} mice receiving more vaccinations had higher or equal IFN-γ- and IL-4-producing cells compared with mice receiving a single vaccination (Fig. 5*c*). Although direct comparison of the absolute protection and CTL activity between C3H and C3H.^{lpr} mice cannot be done, the pattern of antitumor protection, CTL generation, and ELSPOT results suggest that, in Fas receptor-negative mice in C3H background, repeated vaccinations do not have a negative impact on tumor protection and are not associated with a cytokine pattern shift.

DISCUSSION

Cellular immune responses in experimental systems requiring both CD4 and CD8 cells are initiated by antigens presented as peptide epitopes in MHC class II molecules by professional APCs. Antigen-specific CD4 cells recognize class II-presented peptide epitopes and activate the APCs through CD40/CD40-ligand interactions (19–21). CD4 cells and the activated APCs generate a cytokine pattern that will modulate the effector function of CD8 cells recognizing epitopes derived from the same antigen presented by MHC class I molecules on the activated APCs. Cytolytic T cell responses are thought to be enhanced by the production of type 1 cytokines such as IFN-γ and

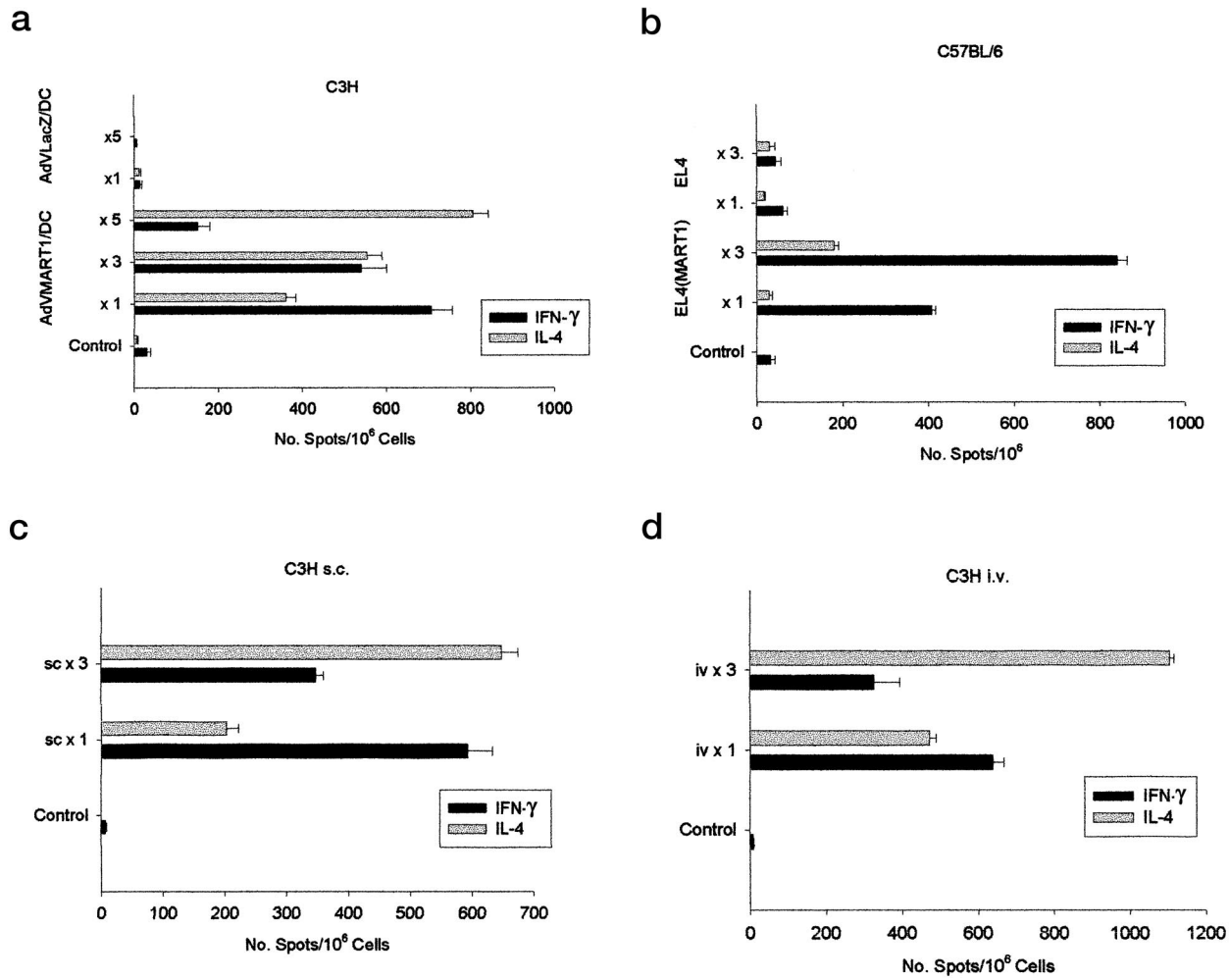

Fig. 3. Type 1/type 2 cytokine-producing cells after repeated DC vaccinations. Animals were immunized, and splenocytes were restimulated *in vitro* for 48 h using the same protocol as described for microcytotoxicity assays. Restimulated splenocytes were added to precoated ELISPOT plates in duplicate 3-fold dilutions. After 24 h, plates were washed and labeled with a secondary antibody. *a*, cytokine-producing cells from C3H mice immunized i.v. with either AdVMART1/DC or AdVLacZ/DC (5×10^5 DCs/mouse). All splenocytes for this assay were restimulated with irradiated SVEC(MART-1). *b*, cytokine-producing cells from C57BL/6 mice immunized s.c. Splenocytes were restimulated either with irradiated EL4(MART-1) or the parental EL4 cell line. In a direct comparison to determine the effect of the route of immunization, cytokine-producing cells from concurrent immunization of C3H mice either s.c. (*c*) or i.v. (*d*) were analyzed. Both groups of splenocytes were derived from mice immunized at the same time and assayed at the same time [SVEC(MART-1) restimulation]. *Bars*, SD.

IL-12, whereas antibody production and diminished cell-mediated responses are associated with type 2 cytokine profiles with the production of IL-4, IL-5, and/or IL-10 (17). In our model, DCs endogenously expressing high levels of a well-characterized melanoma antigen were used as APCs to prime antigen-specific immune responses. Multiple vaccinations in C3H mice induced a reproducible decrease in tumor protection. Possible explanations for these findings include antiadenoviral responses that interfere with the generation of tumor-specific immunity, immune deviation [in which the cytokine-responsive T cells shift from an environment that supports cell-mediated tumor destruction (type 1) to a response that does not (type 2)], and clonal deletion (in which antigen-specific T cells are eliminated or exhausted after a strong antigenic stimulation).

Although antiadenoviral humoral and cellular responses are generated in mice vaccinated with adenovirally transduced DCs, these do not seem to significantly alter the ability to subsequently generate anti-MART-1 cellular immunity in mice immunized with AdVMART1/DC. In our studies, a single AdVMART1/DC vaccination after repeated adenoviral exposures (in the form of DCs transduced with a type-matched adenoviral vector without the *MART-1* gene) was able to generate similar protection compared with naive mice receiving a single AdVMART1/DC vaccination. This was achieved despite the presence of higher titers of antiadenoviral neutralizing antibodies in mice receiving multiple AdVMART1/DC administrations. Other studies have also documented that adenoviral epitope presentation by adenovirally transduced DCs expressing model tumor antigens do not generate competitive antiadenoviral immune responses that would preclude the recognition of the immunizing tumor antigen (22, 23).

Deviation from a type 1 to a type 2 cytokine profile has been associated with poorer antitumor protection. This is mediated by the inhibition of the effector function and the generation of an opposing immune response to the same antigen (24). In our model, the poorer antitumor protection *in vivo* and the decreased lytic activity *in vitro* are associated with a shift in the cytokine pattern: C3H mice receiving multiple vaccinations had more type 2 cytokine-producing cells, whereas mice immunized only once had higher numbers of type 1 cytokine-producing cells. However, C57BL/6 mice did not show this pattern of cytokine response. Increased antitumor protection with multiple vaccinations in C57BL/6 mice was associated with an increase in IFN-γ production. Previous studies using models for infectious and autoimmune diseases have also shown a mouse strain-related cytokine response to the same antigen. C57BL/6 mice infected with *Leishmania major* produce high levels of IFN-γ, generate a

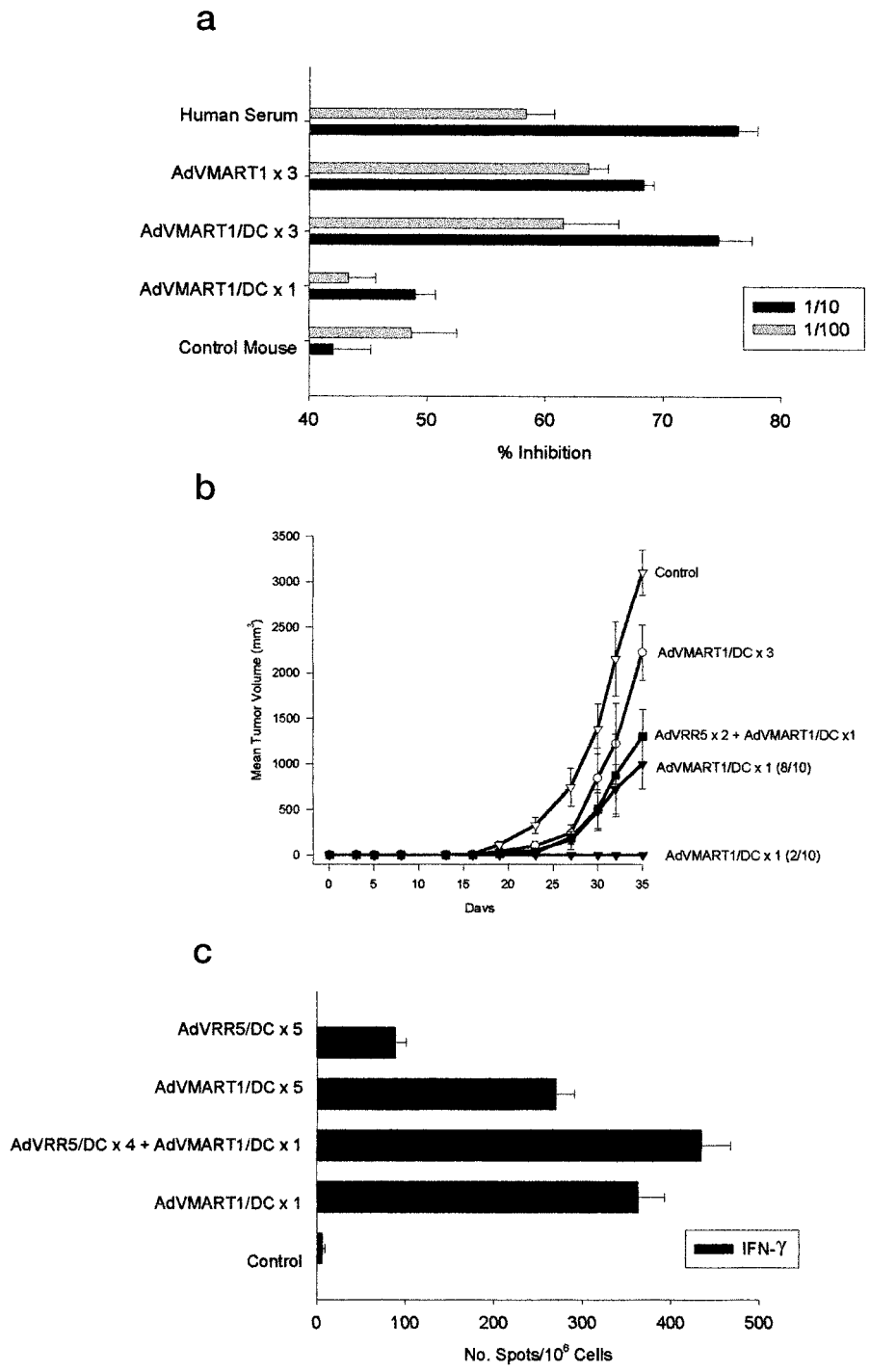

Fig. 4. Antiviral responses in C3H mice immunized with adenovirally transduced DC. For humoral responses, the presence of antiviral neutralizing antibodies was assessed in mice immunized three times with i.p. injection of AdVMART1 or in mice immunized once or three times with AdVMART1/DC. The presence of antibodies to the adenoviral backbone was assessed by incubating serial dilutions of serum obtained from mice (two/group) with the type-matched vector AdVLacZ for 1 h. Similar results were observed after transducing HeLa cells and staining for β-galactosidase (not shown) and transducing 293 cells and counting plaque formation (*a*). Each study has been repeated twice with similar results. For cellular responses, NFSA(MART-1) tumor growth was assessed in C3H mice immunized twice with DCs transduced with the "empty" AdVRR5 replication-deficient adenoviral vector and once with AdVMART1/DC (■) and compared with mice immunized once (▼) or three times (○) with AdVMART1/DC or that were not immunized (▽) (*b*). The pattern of IFN-γ production after repeated AdVRR5 with a final AdVMART1/DC vaccination was compared with the same total number of AdVRR5/DC or AdVMART1/DC vaccinations or with a single AdVMART1/DC vaccination (*c*). *Bars,* SD.

strong protective T-cell response, and do not develop infection. Conversely, BALB/c mice produce minimal levels of IFN-γ and T-cell responses to *Leishmania* antigens, which leads to progressive disease and death (25). Moreover, antigen-specific IL-4 production is higher in the susceptible BALB/c mice than in the *Leishmania*-resistant C57BL/6 mice (26). Similarly, B10.D2 mice exposed to HA produce type 1 cytokines, whereas BALB/c mice produce type 2 cytokines. These results correlate with the observation that double-transgenic mice expressing influenza HA under the rat insulin promoter in the islet β cells of the pancreas and a T-cell receptor transgene specific for HA on a B10.D2 background are diabetes prone, whereas similar double-transgenic mice on a BALB/c background are diabetes resistant because of diminished cell-mediated responses associated with the type 2 phenotype (27). Although this strain-specific immune deviation has not been described previously for tumor antigens, our data suggest a similar pattern of differential type 1/type 2 responses generated by a model tumor antigen, which correlates with *in vivo* and *in vitro* antitumor effects.

Clonal deletion of MART-1-specific activated lymphocytes may be involved in the generation of poorer protection with multiple vaccinations in C3H mice. In a transgenic mouse model in which the T-cell receptor is specific for an antigen expressed by mouse plasmacytoma cells, clonal deletion of antigen-specific T cells was demonstrated with increasing antigen exposure (28). This effect was attributable to

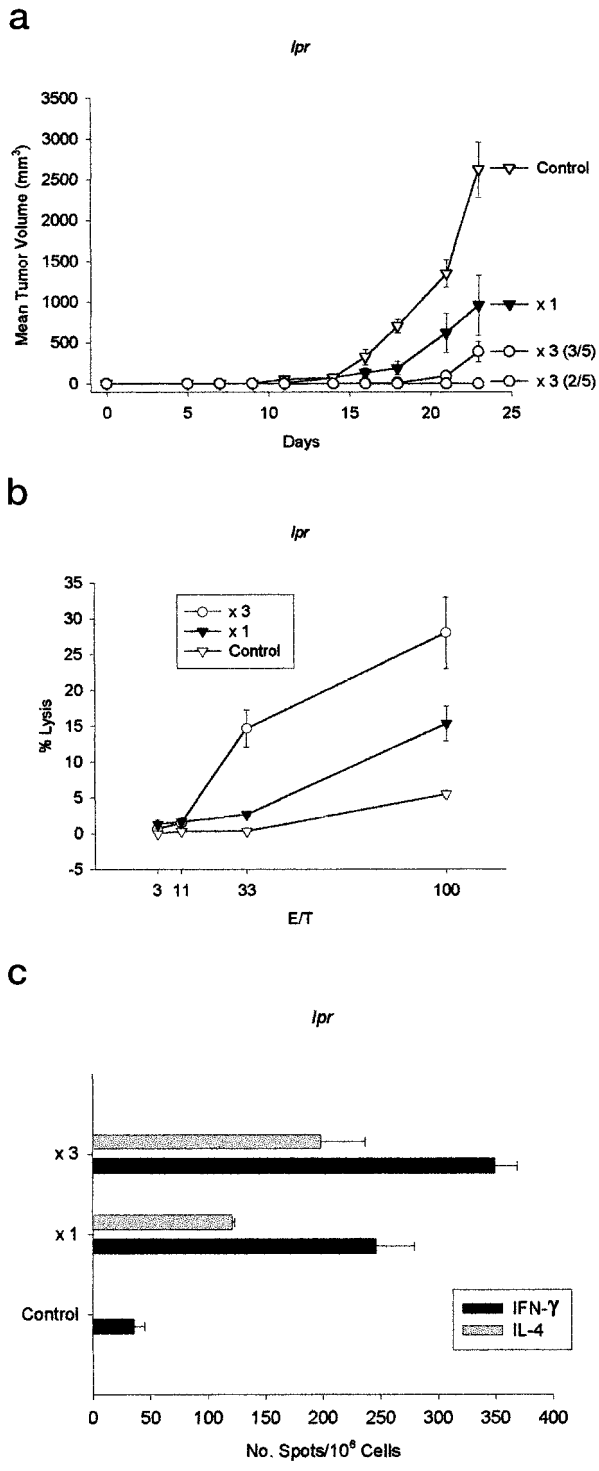

Fig. 5. *In vivo* and *in vitro* antitumor protection with AdVMART1/DC in Fas receptor-negative C3H.^{lpr} mice. *a*, C3H.^{lpr} mice were immunized i.v. once (▼) or three times (○) with AdVMART1/DC and challenged 10 days later with NFSA(MART-1) cells obtained from progressively growing tumors in C3H mice. Mice immunized three times showed a trend toward better antitumor protection ($P = 0.07$), which was consistent in a replicate study. Splenocytes from similarly immunized mice were harvested after 10–14 days and restimulated *in vitro* with irradiated SVEC(MART-1) to be used in microcytotoxicity assays (*b*) or ELISPOT assays (*c*). Results are representative of at least three similar studies for each assay. *Bars*, SD.

the processing of circulating myeloma protein by APCs, leading to the deletion of tumor-specific lymphocytes (11). The mechanism responsible for the clonal deletion of activated T cells may be Fas-mediated AICD, a process implicated in the regulation of immune responses (29). It has been shown that stimulation of previously activated T cells results in the expression of Fas ligand mRNA and increased ability to lyse Fas receptor-positive targets (30). Mice carrying the C3H.^{lpr} phenotype develop massive generalized lymph node involvement, which is attributable to a defect in Fas-mediated lysis of self-reacting cells (31). These mice were used to determine whether Fas-mediated AICD was involved in clonal deletion of MART-1-specific lymphocytes in C3H mice. C3H.^{lpr} mice receiving multiple vaccinations demonstrated better antitumor protection, higher target cell lysis *in vitro*, and no shift in cytokine pattern. Although these data are suggestive of a role of Fas-mediated AICD in this model, the contribution of non-Fas killing mechanisms (tumor necrosis factor, tumor necrosis factor-related apoptosis-inducing ligand, CD30, and Nur-77; Refs. 32 and 33) or other modulators [like subpopulations of DCs present in the vaccination (34, 35), type of B7 costimulation (36), IL-2 (37), IL-15 (38), calcium, sphingomyelins, or corticosteroids (39)] cannot be ruled out.

Because AICD is a potent form of immune regulation, differential sensitivity of the type 1/type 2 cell subsets to undergo apoptotic death after activation may explain our findings (40). In transgenic mouse models and using T-cell clones, in which a large proportion of cells are specific for a strong foreign antigen, several reports have demonstrated that type 1 cells are more sensitive and undergo more rapid apoptotic death after stimulation, whereas type 2 cells are relatively resistant to AICD (40, 41). Differential AICD in type 1/type 2 cells is mainly mediated by Fas/Fas ligand and is in part attributable to a Fas-associated phosphatase-1 protective effect in type 2 cells (42). The preferential survival of type 2 cells in mixed cultures stimulated by antigen leads to detection of a type 2 cytokine pattern (43). Therefore, this difference in susceptibility to Fas-induced AICD could explain the polarized type 2 response seen with multiple vaccinations in C3H mice but absent in C3H.^{lpr} mice on the same background. However, we were unable to directly detect this effect because the frequency of antigen-specific T-cell subsets in our models was too low to determine their apoptotic sensitivity.

How human subjects will respond to tumor antigen vaccination delivered by DCs is currently unknown. Early results of clinical trials in lymphoma and melanoma are encouraging (7, 8). These trials, as well as many others currently underway, usually call for multiple DC vaccinations with measurement of clinical and immunological end points. Our findings challenge the existing paradigm in clinical immunotherapy that more immunizations are better. They underscore the importance of understanding the biology of responses generated by performing serial measurements of immune responses after each DC vaccination in clinical trials. It is possible that responses in cancer patients may vary according to HLA alleles, tumor antigens, or non-MHC genes that regulate the T-cell physiology (27).

In conclusion, the immunological and antitumor effects of genetic immunization with tumor antigen-expressing, genetically modified DCs are dependent on genetic background. When MART-1 is used as a model tumor antigen in mice, both immune deviation and clonal deletion of activated cells may play a role in modulating this response, which results in either increased or decreased antitumor protection *in vivo* after multiple DC vaccinations.

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