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Enhanced Tumor Responses to Dendritic Cells in the Absence of CD8-Positive Cells†

Antoni Ribas,‡† Jennifer A. Wargo,‡ Begonya Comin-Anduix,‡ Shelley Sanetti,* Lana Y. Schumacher,‡ Colin McLean,‡ Vivian B. Dissette,‡ John A. Glaspy,* William H. McBride,‡ Lisa H. Butterfield,‡ and James S. Economou‡§

Wild-type mice immunized with MART-1 melanoma Ag-engineered dendritic cells (DC) generate strong Ag-specific immunity that has an absolute requirement for both CD8⁺ and CD4⁺ T cells. DC administration to CD8α knockout mice displayed unexpectedly enhanced levels of protection to tumor challenge despite this deficiency in CD8⁺ T cells and the inability to mount MHC class I-restricted immune responses. This model has the following features: 1) antitumor protection is Ag independent; 2) had an enhanced levels of protection to tumor challenge despite this deficiency in CD8⁺ T cells; 3) CD4⁺ splenocytes are responsible for cytokine production; 4) lytic cells in microcytotoxicity assays express NK, but lack T cell markers (NK1.1⁺/CD3⁺/CD8⁻/TcRαβ⁻); and 5) the lytic phenotype can be transferred to naive CD8α knockout mice by NK1.1⁺ splenocytes. Elucidation of the signaling events that activate these effective cytotoxic cells and the putative suppressive mechanisms in a wild-type environment may provide means to enhance the clinical activity of DC-based approaches. The Journal of Immunology, 2004, 172: 4762–4769.

Epitopes presented through MHC class I and II are recognized by CD8⁺ and CD4⁺ T cells, respectively, which work together to generate efficient Ag-specific antitumor responses. Depletion of either one of these two cellular subsets leads to complete abrogation of gene-modified DC-induced immune responses in the majority of models (reviewed in Ref. 2), confirming that both immune cell subsets are required for the response. The requirement of CD4⁺ T cells can be bypassed by CD40 cross-linking of the gene-modified DC, and the immune response in a CD4-null environment generated by CD40-engaged DC is abrogated when CD8⁺ T cells are depleted (7). Therefore, the emerging picture suggests that gene-modified DC present Ag through MHC class II to CD4⁺ Th cells, which engage the CD40 receptor on the DC leading to activation of effector CD8⁺ CTL recognizing cognate Ag presented by MHC class I molecules.

CD8α knockout (CD8αKO) mice have no detectable MHC class I-restricted responses (8, 9), and have been widely used to study the role of CD8⁺ T cells in immune responses. The CD8 molecule is a disulfide-linked transmembrane heterodimer of α- and β-chains. The cytoplasmic tail of the CD8 α-chain associates with intracellular tyrosine kinases critically involved in TCR-mediated signaling (10). CD8 β-chain lacks motifs for interacting with known signaling molecules, and in the absence of CD8 α-chains does not reach the cell surface. Therefore, the CD8 β-chain cannot replace the absence of CD8 α-chains, and homodimers of CD8β are not expressed in CD8αKO mice.

In our previous experience, CD8 Ab depletion completely abrogated antitumor responses induced by immunization with DC transduced using an adenovirus encoding the MART-1 melanoma Ag (AdVMART1) in wild-type C3H and C57BL/6 mice (7, 11, 12). However, when we extended these studies to CD8αKO mice, an unexpected observation of markedly improved protection to a B16 tumor challenge was consistently noted. In this work, we characterize the immunobiology of this effective antitumor response, which is mediated by an interaction between lytic NK cells and cytokine-producing CD4⁺ T cells. The antitumor activity of these DC-activated cells efficiently surpasses that which can be achieved in a wild-type, CD8⁺ environment.

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2 Address correspondence and reprint requests to Dr. Antoni Ribas, Division of Hematology-Oncology, 11-934 Factor Building, University of California Medical Center, 10833 Le Conte Avenue, Los Angeles, CA 90095-1782. E-mail address: aribus@mednet.ucla.edu

3 Abbreviations used in this paper: DC, dendritic cell; AdVMART1, replication-deficient adenoviral vector encoding MART-1 melanoma Ag; KO, knockout.
Materials and Methods

**Cell lines and recombinant adenoviral vectors**

B16, a murine melanoma; EL4, a murine lymphoma cell line; and 3LL, a murine lung carcinoma, were obtained from the American Type Culture Collection (Manassas, VA). B16 and EL4 were maintained in vitro in DMEM (Life Technologies) and 3LL in RPMI 1640, in both cases supplemented with 10% FCS (Gemini Bio-Products, Calabasas, CA) and 1% (v/v) penicillin, streptomycin, and amphotericin (Gemini Bio-Products). Generation of EL4 and 3LL MART-1 and NeoR stable transfectants has been previously described (12). Stably transfected cells were maintained in vitro under constant G418 selection (0.5 mg/ml; Life Technologies, Rockville, MD). AdVMART1 and AdVLacZ are E1-deleted replication-deficient adenoviral vectors based on human type 5 adenoviruses. The construction and characterization of these vectors have been described previously (11, 13). The transgenes are driven by the human CMV early promoter/enhancer.

**Preparation of DC and adenoviral transduction**

DC were differentiated from bone marrow progenitor cells derived from C57BL/6 or C57BgrKO mice by in vitro culture in GM-CSF and IL-4, as described previously (14) with modifications (13). DC were harvested as loosely adherent cells and transduced in RPMI 1640 with 2% FCS transduction medium at a multiplicity of infection of 100 viral PFU per each DC when the final volume was 1 ml, and a multiplicity of infection of 200 when the final volume was 2 ml. Transduction was conducted for 2 h at 37°C, after which time 5 vol of 10% FCS RPMI 1640 medium was added to neutralize free virus, and then the AdV-transduced DC were washed twice in PBS and resuspended in 0.2 ml of PBS per animal for injection. Transduction efficiency of murine DC using these procedures is >70% (11, 13). After assessment of the lack of contribution of the MART-1 Ag or the adenoviral transduction to the protective phenotype generated by DC administration, groups of mice receiving DC with or without adenoviral vector transduction were pooled for data analysis.

**Mice and confirmation of CD8α deficiency in CD8rKO mice**

C57BL/6 and CD8 α-chain KO mice in a C57BL/6 background (B6.129S2-CD8atm1Mak, backcrossed >28 generations) were purchased from The Jackson Laboratory (Bar Harbor, ME) and were bred and kept under specific pathogen-free conditions at the Animal Facility of the Division of Experimental Radiation Oncology, University of California, Los Angeles. Mice were handled in accordance with the University of California animal care policy. Five- to 8-wk-old female or male mice were used, with each study designating gender. CD8rKO mice used in the current studies were confirmed to be genetically deficient of CD8α by flow cytometry. To rule out the possibility of being leaky for the CD8 α-chain or that CD8 β-chain homodimers substituted the genetic deficiency in CD8 α-chains, splenocytes from 4-wk- to 4-mo-old, unimmunized or DC-immunized CD8αKO mice were stained with CD8 α (clone 53-67; BD Pharmingen, San Diego, CA)- and CD8 β (clone 53-58; BD Pharmingen)-chain Abs by flow cytometry. This was compared with splenocytes from wild-type C57BL/6 mice.

**Skin graft transplantation**

Mice were anesthetized with i.p. ketamine (200 mg/kg) and xylazine (10 mg/kg). Full-thickness (0.5 × 0.5 cm) skin was harvested from a shaved area of the dorsal skin. The graft was fixed to the recipient mouse with sutures of 6-0 silk thread. Reaction was defined as a totally necrotic graft.

**Animal studies**

Mice were immunized on days 1 and 8 with 1–5 × 10^5 DC/mouse administered s.c. in the right flank, and challenged on the left flank 10–14 days after the last immunization with B16 or 3LL stably transfected with MART-1 or NeoR mock-transfected cells (7–10 × 10^5 cells/animal). B16 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 medium- and serum-derived epitopes. To generate single cell suspensions, tumors were surgically removed, decapsulated, and minced. Minced tumors underwent enzymatic digestion for 1–2 h with Dnase I (0.1 mg/ml; Sigma-Aldrich, St. Louis, MO) and collagenase D (1 mg/ml; Boehringer Mannheim, Indianapolis, IN) in 50 ml of AIM-V medium (Life Technologies). Viable cells were washed three times in PBS and resuspended in 0.2 ml of PBS per animal to be injected s.c. into the left flank. Injected cells were >70% viable, as determined by trypan blue exclusion (11–13). There was no difference in protection when using B16 tumors harvested from CD8αKO or C57BL/6 mice (data not shown), and tumors for passage were always harvested from non-DC-administered mice. Each treatment group typically contained five mice for in vivo tumor challenge studies and one mouse to obtain splenocytes for in vitro studies (ELISPOT, 51Cr release assays).

**In vivo Ab treatment**

In vivo mAb ablation of CD8α (clone 2.43), CD4α (clone GK1.5) T cell subsets, and NK1.1α (clone PK136) cells was performed by i.p. injection of 100 μg of purified endotoxin-free Ab/injection (BD Pharmingen) on days −5, −3, and −1 before tumor inoculation, and every 6 days thereafter. Monitoring of successful depletion was performed by flow cytometry on splenocytes harvested on the day of tumor inoculation for CD4- and CD8-depleting Abs. 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 microcytotoxicity assays, splenocytes were harvested 14 days after the last immunization, depleted of RBC by hypotonic lysis, cultured in vitro with or without irradiated EL4 or EL4(MART-1) at a 25:1 ratio, or no stimulator cells (as indicated) for 96 h, in all cases in the presence of 10 U/ml IL-2, and assayed in a standard 4-h chromium release test. After assessment of the lack of contribution of the restimulator cells or the MART-1 Ag in the generation of lytic effector cells, subsequent studies were performed with RBC-depleted splenocytes kept in complete medium in the presence of 10 U/ml IL-2 for 96 h.

**Cytokine profile by ELISPOT**

RBC-depleted splenocytes, cultured in vitro for 48 h 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 Ab (BD Pharmingen) and incubated for 24 h at 37°C, as previously described (12, 15–17). Spots were developed using a secondary biotin-labeled Ab and counted under a dissecting microscope.

**In vitro cellular subset selection**

In selected studies, splenocytes were positively or negatively selected for CD3+, CD4+, NK1.1+, and αβTCR+ cells using magnetic columns (Miltenyi Biotec, Auburn, CA), following the manufacturer’s instructions. Successful cell subset isolation or depletion was monitored by flow cytometry comparing pre- and postcolumn samples.

**Adoptive transfer studies**

Splenocytes from AdVMART1/DC-immunized CD8αKO mice were harvested, depleted of RBC, and prepared for reinfusion without an ex vivo stimulation step. In selected studies, splenocytes underwent NK1.1 selection using magnetic columns (Miltenyi Biotec). Splenocytes were washed twice in PBS, and 5 × 10^6 cells/mouse in 0.2 ml of PBS were injected i.v. One week later, spleens of host mice receiving splenocyte adoptive transfer were harvested and used in microcytotoxicity assays.

**Statistical analysis**

Results of in vivo studies are presented as the mean and SEM of tumor volumes in each treatment group, or as the time to first tumor development. In tumor volume graphs, 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 (13). Treatment groups divided into mice with and without tumors in a tumor volume plot are noted with the number of mice with (or without) tumor from that group over the total number of mice in the group, which is shown in parentheses in the graph. Significance in tumor volume graphs, number of IFN-γ-producing cells in ELISPOT assays, and lytic activity in microcytotoxicity assays is calculated using the t test. Graphs of tumor development over time are presented using Kaplan-Meier plots, with the significance calculated using the log-rank test.
Results

Superior protection to B16 in CD8αKO mice

Protective B16-specific antigen responses after immunization with AdVMART1 gene-modified DC in C57BL/6 wild-type mice are abrogated by Ab-mediated depletion of either CD8α or CD4+ T cell populations (7, 12, 18). The contribution of CD4+ T cells can be bypassed by DC maturation through CD40 cross-linking; depletion of CD8α T cells in this setting abrogates the response (7), indicating that MHC class I-restricted CD8+ T cells are the critical effectors in the wild-type environment.

We decided to immunize CD8αKO mice with the goal of studying pure CD4 responses to AdVMART1/DC vaccination. CD8αKO and wild-type C57BL/6 mice were immunized with two weekly s.c. injections of DC-based vaccines and then challenged 10–14 days later with the MART-1-positive murine melanoma B16. Cells for tumor challenge were obtained from progressively growing s.c. tumors in syngeneic mice, and therefore not exposed to xenogenic antigens derived from bovine serum. Fig. 1 shows a representative tumor growth experiment, with unimmunized C57BL/6 wild-type and CD8αKO mice all developing rapidly progressing B16 tumors. Consistent with what we have previously described (7, 12, 17, 18), wild-type C57BL/6 mice immunized with AdVMART1/DC had partial or complete protection to a B16 tumor challenge, which was abrogated by CD8 Ab-mediated depletion. CD8αKO mice immunized with AdVMART1/DC actually had superior levels of protection to B16 tumor challenge compared with wild-type mice. Moreover, the administration of CD8-depleting Ab had no effect on protection (p = 0.01 compared with control mice). Thus, cells expressing the CD8 phenotypic marker, essential for protection in wild-type mice, are neither present nor required in CD8α genetically deficient mice. A cumulative analysis of 17 independent studies using a total of 139 wild-type and 187 CD8αKO mice confirmed higher levels of protection to B16 in CD8αKO compared with wild-type mice after DC administration (70 vs 30.5%, p < 0.0001).

Confirmation of CD8α deficiency in CD8αKO mice and lack of minor histocompatibility mismatch between wild-type and CD8αKO mice

CD8αKO mice were generated by Mak and colleagues (8, 9) by targeted disruption in D3 embryonic stem cells from 129/Sv mice, which were injected into C57BL/6 blastocysts to generate the chimeric mice. Mice backcrossed for >28 generations were obtained from The Jackson Laboratory. To confirm their CD8αKO phenotype, splenocytes were stained for flow cytometry. There were no detectable surface CD8α- or CD8β-chains on splenocytes from CD8αKO mice at any age tested (the CD8 β-chain requires the presence of CD8 α-chains to make functional heterodimers), while splenocytes from wild-type mice were positive for both CD8α- and CD8β-chains (data not shown). Because the original embryonic cell line was derived from 129/Sv mice, there was the possibility of residual minor histocompatibility mismatch between C57BL/6 mice and CD8αKO mice despite the extensive backcrossing. This was ruled out by the acceptance of reciprocal skin grafts between wild-type and CD8αKO mice in C57BL/6 background (data not shown).

Ag nonspecificity of protection

This protection in CD8αKO mice appears to be an Ag-independent and transduction-independent phenomenon. Mice receiving DC transduced with AdVLacZ or untransduced DC exhibited the same degree of protection to a B16 tumor challenge (Fig. 2A; p = 0.008 when comparing any of the three DC-administered groups with the control group). Ag nonspecificity was reinforced in experiments in which mice received AdVMART1/DC and were also challenged with the Lewis lung carcinoma (3LL) cell line stably transfected with MART-1 or a control selectable marker (Neo). Cumulative tumor development curves from two independent studies in mice challenged with 3LL(MART1) or 3LL(Neo) demonstrate that there was no difference in in vivo protection (Fig. 2B). Therefore, DC administration, and not adenoviral vector transduction nor the engineering with a defined tumor Ag, is responsible for the protective phenotype.

NK1.1+ and CD4+ cells are required for the in vivo protection in CD8αKO mice

We then studied the contribution of immune cell subsets in the response observed in CD8αKO mice. Depletion of NK1.1+ cells (PK136 hybridoma; Fig. 3A) or CD4+ T cells (GK1.5 hybridoma; Fig. 3B) led to abrogation of protection to B16. NK1.1 depletion led to a complete loss of the protective phenotype (p = 0.014; Fig. 3A), while depletion of CD4+ T cells still allowed a 1-wk delay in tumor outgrowth (p = 0.006; Fig. 3B). Splenocytes from DC-sensitized CD8αKO mice produced IFN-γ in ELISPOT assays and were cytotoxic for B16 in microcytotoxicity assays (see Figs. 4a and 6a), but could not be demonstrated in NK1.1- or CD4-depleted mice (data not shown). These reproducible findings suggest to us that cells bearing the NK1.1 marker can provide only partial tumor suppression by themselves in DC-sensitized CD8αKO mice, and collaborate in some manner with CD4+ cells to effect complete protection. The steady state or after DC administration numbers of CD4+ and NK1.1+ splenocytes were the same in CD8αKO and C57BL/6 mice, evaluated by flow cytometry (data not shown).
suggested that the number of responsive cells was not responsible for the difference in protection.

CD4+ cells produce IFN-γ in an Ag-independent manner after DC administration to CD8αKO mice

Splenocytes from CD8αKO mice receiving DC s.c. administrations were restimulated in 10 U/ml IL-2 with MART-1-transfected or negative EL4 cells, or without restimulator cells, and cells producing IFN-γ were enumerated using ELISPOT assays. In a series of seven studies, wild-type and CD8αKO mice received DC with or without MART-1 gene modification and were cultured in the presence or absence of restimulator cells. Wild-type mice immunized with AdVMART1/DC generated an Ag-specific response, with significant differences when splenocytes were restimulated with MART-1-positive or negative EL4 (p = 0.00012; Fig. 4A), while the number of IFN-γ-producing cells in the absence of restimulator cells was close to background. On the contrary, DC-sensitized splenocytes from CD8αKO mice produced the same level of IFN-γ production regardless of the presence or absence of MART-1-expressing restimulator cells (p not significant; Fig. 4A), again suggesting that this is an Ag-independent response. Depletion of CD4+ T cells before adding the splenocytes to the

ELISPOT plate removed the IFN-γ-producing cells. Addition of purified CD4+ T cells completely restored the ability to produce IFN-γ in this assay. No difference in the number of cytokine-producing cells was noted when NK1.1+ cells were depleted (Fig. 4B). In summary, the IFN-γ response in CD8αKO mice was Ag nonspecific, and production of this cytokine is entirely dependent on CD4+ T cells.

Cells with an NK phenotype are responsible for the lytic activity in vitro

We attempted to define the contribution of NK1.1+ lytic cells in vitro by subset isolation before microcytotoxicity assays. NK1.1+ cells may be true NK cells, CD1-restricted NKT cells, QA-1-restricted NKT cells, or TCR-restricted CTLs, or TCR-restricted CTLs with constitutive or acquired NK1.1 expression (NK1.1+ CD8 wannabes) (19–22). For these assays, splenocytes obtained from DC-administered CD8αKO mice were run through NK1.1, CD3, and αβ TCR magnetic isolation columns before mixing with chromated B16 cells. Successful depletion and cell isolation were monitored by flow cytometry (data not shown). NK1.1+ cells alone were sufficient to lyse B16 in vitro, while the NK1.1-negative cell fraction had decreased lytic activity (Fig. 5A). When cells were separated using CD3 and αβ TCR columns (Fig. 5, B and C), only cells without these two T cell markers maintained lytic activity. Therefore, lytic cells in vitro have a NK1.1+ αβ TCR− CD3− phenotype.
NK1.1<sup>+</sup>-activated splenocytes from CD8αKO mice maintain lytic activity when transferred to naive CD8αKO mice, but not to naive C57BL/6 mice

To further define the population responsible for the protective phenotype, splenocytes from DC-activated CD8αKO mice were adoptively transferred into naive host CD8αKO or C57BL/6 wild-type mice. These splenocytes could transfer lytic activity to B16 when injected into naive CD8αKO mice, but not to C57BL/6 wild-type mice (p = 0.0004; Fig. 6A), suggesting that wild-type mice have a negative regulatory mechanism that inhibits the lytic activity of the transferred cells. To determine whether CD8<sup>+</sup> splenocytes from wild-type mice were a suppressive population inhibiting the stimulatory actions of DC in CD8αKO mice, CD8αKO mice received i.v. adoptive transfer of CD8αKO splenocytes from wild-type mice (5 x 10<sup>6</sup> cells/mouse) and then received DC s.c. 4 days later. Spleens were harvested 2 wk later and assayed by ELISPOT and 51 Cr release assays. There was no difference in the number of IFN-γ-producing cells (Fig. 6B; p nonsignificant comparing addition of CD8<sup>+</sup> or CD8<sup>−</sup> splenocytes from wild-type mice) or lytic activity to B16 (Fig. 6C; p nonsignificant) regardless of receiving or not receiving CD8<sup>+</sup> splenocytes from wild-type mice i.v. Therefore, we were unable to detect a suppressive effect of CD8<sup>+</sup> T cells from wild-type mice at the tested conditions.

To determine which cell subset was responsible for IFN-γ production and lytic activity in recipient CD8αKO mice, splenocytes from DC-activated CD8αKO mice were positively or negatively selected for NK1.1<sup>+</sup> cells by passage through magnetic columns and then adoptively transferred i.v. to naive CD8αKO mice. The flow through and eluted cell populations were confirmed to contain the relevant subpopulation by flow cytometry, routinely with 5% contaminating cells (data not shown). Depletion of NK1.1<sup>+</sup> from the donor splenocytes decreased the number of IFN-γ-producing cells in the recipient mouse splenocytes assessed by ELISPOT assays, while reconstitution with DC-activated NK1.1<sup>+</sup> cells alone was sufficient to adoptively transfer the ability to generate IFN-γ to host CD8αKO mice (Fig. 7A). Adoptive transfer of NK1.1<sup>+</sup> cells alone also resulted in transfer of in vitro lytic activity to B16, at levels equivalent to the transfer of whole splenocytes from DC-administered CD8αKO mice (Fig. 7B). In agreement with the results of ELISPOT assays, depletion of NK1.1<sup>+</sup> from
comparing lytic activity from NK/H11001/H11001/H9251/H11001 but not naive C57BL/6 wild-type mice, without detectable inhibition by CD8 using B16 as tumor target (H9251). This study has been repeated three times with ex vivo activation) from DC-activated CD8/H11001/H9251. Successful splenocyte adoptive transfer to naive CD8/H9251/H9253 weeks later, splenocytes were harvested and assayed for the number of IFN-producer cells was able to transfer splenic IFN-γ-producing cells by ELISPOT (B) or lytic activity in microcytotoxicity assay using B16 as tumor target (C).

**FIGURE 6.** Successful splenocyte adoptive transfer to naive CD8αKO, but not naive C57BL/6 wild-type mice, without detectable inhibition by CD8 T cells from wild-type mice adoptively transferred to CD8αKO mice. A, Adoptive transfer of splenocytes (5 x 10⁶ RBC-depleted splenocytes injected i.v., without ex vivo activation) from DC-activated CD8αKO mice confers lytic activity in naive CD8αKO, but not wild-type mice. Microcytotoxicity assay using B16 as tumor target. This study has been repeated three times with similar results. B and C, Lack of detectable suppressive effect of CD8 T cells from wild-type mice administered to CD8αKO mice. CD8αKO mice received adoptive transfer of CD8⁺ or CD8⁻ splenocytes (5 x 10⁶ injected i.v.) from naive C57BL/6 mice, followed 4 days later by s.c. DC administration. Two weeks later, splenocytes were harvested and assayed for the number of IFN-γ-producing cells by ELISPOT (B) or lytic activity in microcytotoxicity assay using B16 as tumor target (C).

Discussion

Administration of DC to CD8αKO mice induces an effective Ag-independent protective response to B16, which suggests that CD8α genetically deficient mice have developed a DC-stimulated compensatory mechanism that efficiently bypasses the need for CD8⁺ CTL. The in vivo protection is independent of FCS-mediated epitopes, because the cells used for tumor challenge were derived from syngeneic mice and not exposed to culture medium. Our data suggest that cells with an NK phenotype play a major role in the antitumor response in CD8αKO mice, and in collaboration with CD4⁺ Th cells they provide complete protection in most animals. We have also confirmed these findings of increased protection in a CD8 genetically deficient environment using β₂-microglobulin KO mice (23), which lack MHC class I molecules (24).

Murine studies showed that DC can stimulate NK cells, leading to in vivo antitumor activity to NK-preferred (MHC low) tumor cells (25). The NK-DC interaction was shown to be contact dependent and resulted in activation of both NK lytic activity and IFN-γ production (25). Subsequent studies have confirmed these observations in human in vitro cell cultures (26–30). Additional studies focused on the possible role of NK cells in the activation of Ag-specific CTL responses generated by tumor Ag-loaded DC. Studies in both murine and human model systems showed that NK cells contributed to CTL generation (31, 32). The majority of reports on the immunological mechanism of response to tumor Ag gene-modified DC vaccines have shown that both CD4⁺ and CD8⁺ T cells are critically involved (reviewed in Ref. 2). Protection in the absence of CD8 T cells has been reported in one model, although there has been no observation of enhanced immunity in the CD8 genetically null environment (33). Similarly, the contribution of NK cells to the protective response after tumor Ag gene-modified DC vaccines has been infrequently reported, although it has been reported that NK1.1 depletion partially abrogated protective responses in two models (33, 34). Therefore, a two or three cell interaction may be involved in the antitumor response generated by gene-modified DC in the wild-type environment.

CD8αKO mice have a homologous recombination in the CD8α locus, leading to absent intracellular and surface expression of CD8 molecules, which has allowed the study of the role of this surface molecule in CTL function (8, 9). These mice provided evidence that the major role of the CD8 molecule is the stabilization of the interaction between the TCR and MHC class I molecules (35). We confirmed that mice used in the current studies were deficient in CD8α and β surface expression; we were unable to detect even small numbers of CD8⁺ cells that could conceivably play a role in this protective phenotype. No significant expression of CD4⁺ TCRhigh T cells with lytic activity is observed in CD8αKO mice compared with wild-type mice (8), suggesting that the CD8α molecule is critically required for CTL generation, and CD8 wannabes (cells with the same function as CD8⁺ CTL, but only missing CD8 molecules on their surface) are not efficiently generated in these mice (19). This is in contrast to the detection of MHC class II-restricted responses in CD4KO mice, which are mediated by CD4⁻ CD8⁻ TCRhigh T cells (CD4 wannabes) (36). However, when the role of the CD8 molecule in thymocyte development was studied using strong xenoreagitens, a population of CD4⁻ TCRhigh T cells with intracellular CD8β (thereby demonstrating a clear CD8 lineage commitment) could be generated under nonphysiologic conditions of high affinity agonistic heteroclitic epitopes (19). Our data suggest that DC are able to stimulate a population of Ag-independent NK1.1⁺ cells, leading to a very effective antitumor protective response, thereby suggesting that CD8αKO mice may allow the direct study of DC-stimulated innate immune responses.

A number of immune effector populations in CD8αKO mice may bear the NK1.1 marker. These include CD1-restricted NKT
cells, Qa-1-restricted NK-CTLs, atypical CD8-negative CTL with NK1.1 expression (CD8 wannabes), and classical NK cells (19–22). NKT and NK-CTLs coexpress TCR and CD3 (characteristic of T cells) and the NK1.1 receptor (characteristic of NK cells), a phenotype distinct from the effector cells in our model (21, 22). The lack of T cell markers in the lytic cells in this model and the Ag nonspecificity also rule out a major contribution by MHC class I-restricted CD8 CTL with NK1.1 expression. The functional phenotype of DC-induced lytic cells in this model suggests that these cells are closely related to NK cells.

CD4+ cells are required in the induction phase of this response, but not in adoptive transfer studies using splenocytes from DC-sensitized mice. The CD4 response is detected by suboptimal protection in their absence in in vivo depletion studies, and the Ag-independent production of stimulatory cytokines in ELISOT assays. Therefore, CD4+ T cells are likely to have a helper role for NK activation after DC administration in this model, although we cannot formally exclude the participation of CD4+ NK1.1+ double-positive NK or NKT cells (20, 21, 37). NK activity requires cytokines that can be produced by CD4+ T cells (IFN, IL-2, IL-15). However, these cytokines can also be produced by other cell subsets, and a direct helper role of CD4+ T cells on NK activation has not been established (37). Our observations of a NK- and CD4-dependent protection in this CD8-deficient environment may allow further study of the CD4-NK interactions after DC stimulation.

AdVMART1/DC-immunized wild-type C57BL/6 mice have inferior protection to B16 compared with CD8αKO mice, which suggests that the DC-responsive cells in CD8αKO mice are not subjected to the same regulatory control as those in wild-type mice. Adoptive transfer of DC-activated CD8αKO splenocytes into naïve CD8αKO mice replicates the surrogate immunological marker of lytic activity, but not when transferred to naïve wild-type mice. The difference between these mice is CD8α cells, which implicates them as a regulatory/inhibitory population in a wild-type setting. These inhibitory cells are likely to be CD8α T cells or inhibitory CD8α APC. A subpopulation of alloreactive T suppressor cells with a CD8α+CD28− phenotype has been described, and their suppressor effect has been attributed to inhibition of CD40-dependent signaling pathways in APC (38). Also, a subset of IL-10-producing CD8α T cells activated by DC has immune-suppressive functions (39). However, addition of putative CD8α splenocytes from wild-type mice to CD8αKO mice, at the conditions tested in Fig. 6, B and C, failed to define a suppressive role of these cells. Further investigation in the mechanism of suboptimal response to DC in wild-type mice and lack of function of adoptively transferred DC-activated splenocytes from CD8αKO mice is ongoing.

It has been long recognized that T cell-deficient mice have enhanced NK responses, and this has been casually linked to T cell-produced TGF-β1, which has the role of limiting innate responses, while adaptive responses become active after certain viral infections (40). Full characterization of the putative CD8α-suppressive cell population in wild-type mice might provide insight on the critical negative regulatory pathway that prevents the development of the same level of strong anti-B16-protective immunity in wild-type mice compared with CD8αKO mice, thereby providing new clues in how to enhance the activity of DC.

**FIGURE 7.** NK1.1+ splenocyte adoptive transfer is sufficient to transfer the ability to generate IFN-γ and B16 lysis to host CD8αKO mice. A, ELISOT analysis of IFN-γ-producing splenocytes after adoptive transfer of splenocytes from DC-activated CD8αKO donor mice to naïve CD8αKO recipient mice, with or without NK1.1 depletion. B, Microcytotoxicity assay with B16 as tumor target and splenocytes from host CD8αKO mice as effector cells, with or without adoptive transfer of NK1.1+ or NK1.1− splenocytes from DC-immunized CD8αKO mice. These studies have been repeated twice with similar results.
In conclusion, we report remarkable tumor protection in response to DC in mice congenitally deficient in CD8+ T cells. Our data support a multicellular response to DC administration in CD8aKO mice, which consist of lytic cells with an NK phenotype and cytokine-producing CD4+ T cells. This is an Ag and adenosine transduction-independent response. Understanding the mechanism of NK cell activation by DC, the implication of CD4+ T cells, their mechanism of target cell recognition and killing, and the nature of the suppressive cells in wild-type environments may allow enhancement of the benefits of DC-based immunotherapy.

References