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Dendritic Cell-Induced Activation of Adaptive and Innate Antitumor Immunity

Leon T. van den Broeke,* Emily Daschbach,* Elaine K. Thomas,‡ Gerda Andringa,† and Jay A. Berzofsky*‡

While studying Ag-pulsed syngeneic dendritic cell (DC) immunization, we discovered that surprisingly, unpulsed DCs induced protection against tumor lung metastases resulting from i.v. injection of a syngeneic BALB/c colon carcinoma CT26 or a syngeneic C57BL/6 lung carcinoma LL/2. Splenocytes or immature splenic DCs did not protect. The protection was mediated by NK cells, in that it was abrogated by treatment with anti-asialo-GM1 but not anti-CD8, and was induced by CD1−/− DCs unable to stimulate NKT cells, but did not occur in beige mice lacking NK cells. Protection correlated with increased NK activity, and increased infiltration of NK but not CD8+ cells in lungs of tumor-bearing mice. Protection depended on the presence of costimulatory molecules CD80, CD86, and CD40 on the DCs, but surprisingly did not require DCs that could make IL-12 or IL-15. Unexpectedly, protection sensitive to anti-asialo-GM1 and increased NK activity were still present 14 mo after DC injection. As NK cells lack memory, we found by depletion that CD4+ not CD8+ T cells were required for induction of the NK antitumor response. The role of DCs and CD4+ T cells provides a novel mechanism for NK cell induction and innate immunity against cancer that may have potential in preventing clinical metastases. The Journal of Immunology, 2003, 171: 5842–5852.

Most tumors express mutated or inappropriately expressed, nonmutated tumor-associated Ags (TAAs)3 that often contain CTL epitopes. Yet, the immune system often remains incapable of overcoming the growth potential of the malignant cells. Currently, many approaches have been developed to obtain protective and therapeutic antitumor immunity.

Active immunization strategies for treatment or prevention of tumors generally focus on the elicitation of TAA-specific CD8+ CTL responses, because these have the potential to generate durable and protective immunity including T cell memory. The CTL epitopes are peptides usually 8–10 amino acids long with 2–3 primary anchor residues that interact with self-MHC molecules, while 2–3 alternative amino acid residues bind to the TCR. Yet, active immunizations in the form of peptides, proteins, DNA, either alone or with chemical adjuvants, thus far often fail to obtain the desired immune response. Alternatively, there is considerable interest in the use of dendritic cells (DCs) for the delivery of TAAs. DCs have been recognized as very attractive adjuvants because they represent a specialized APC population with the unique ability to activate naïve CD4+ and CD8+ T cells and sustain primary immune responses.

In addition to the generation of tumor-specific CTLs, DCs have recently been found to be able to activate the innate arm of the immune system as well (1). However, the conditions and underlying mechanisms of this immunostimulatory effect still remain poorly defined. An important member of the innate immune system are the CD3− NK cells, which represent the second major population that can recognize and lyse tumors. In contrast to CD8+ T cells, activation of NK cells is not mediated by a TCR and does not require prior immunization with TAAs. Instead, the balance between positive and negative signals initiated by different target cell ligands determines their activity. Ligation of NK inhibitory receptors, such as murine Ly-49A, -49C, and -49G2, by specific MHC class I allotypes delivers a dominant-negative signal and prevents natural killing (2). On the other hand, activation of NK cells is provided by ligation of activating receptors, like murine Ly-49D and Ly-49H. However, understanding of the nature and significance of NK cell activating receptors is lagging behind that of inhibitory NK receptors (3). The second important effectors of the innate immune system are the NKT cells, a subgroup of T cells that express NK markers. NKT cells recognize CD1d, a class I-like molecule that preferentially presents glycolipid moieties, by interaction with the TCR. Inhibition of experimental tumor metastasis has been found with injection of α-galactosylceramide pulsed DCs (4).

Although for most human cancers TAAs have not been identified, recently we have successfully generated human CTLs against synovial sarcoma by TAA-pulsed DCs in vitro (5). Synovial sarcoma is associated with a chromosomal translocation encoding a TAA that spans the fusion breakpoint. Rhabdomyosarcoma is a more common soft-tissue malignancy and is consistently associated with a unique PAX3-FKHR (2, 13)(q35;q14) translocation. The fusion breakpoint encodes peptides with a binding motif for murine H-2Ld (6), indicating that these peptides can be used as TAAs in a BALB/c mouse model.

The original aim of this study was to assess the ability of TAA pulsed DCs to induce protective immunity against rhabdomyosarcoma and to characterize adaptive as well as innate immune responses. In the course of these studies, we serendipitously discovered that DC vaccination can induce tumor immunity through the...
activation of NK cells as well as the induction of TAA specific CD8+ T cells. We explored the mechanism of this unexpected NK activation and tumor protection mediated simply by the injection of fresh DCs without any TAAs. Protection against tumor was found to be NK cell mediated. Activation of NK cells was CD4+ T cell-dependent and strongly relied on the expression of costimulatory molecules on DCs, but did not require expression of IL-12 or IL-15 by the DCs.

Materials and Methods

Mice

Female BALB/c (H-2d) and C57BL/6 (H-2b) were purchased from Charles River Breeding Laboratories (Frederick, MD). Female C129(B6)-Jhdm4d1 (Jh mutation), BALB/c-knockout (KO) IL12p40 (IL12 mutation), C129(B6)-Rag2<sup>−/−</sup>N12 (recombinant-activating gene (RAG)2 KO), and BALB/c-Tac-Hh1<sup>−/−</sup>N9 (nude) mice were obtained from Taconic Farms (Germantown, NY). Female C57BL/6-Ly5<sup>−/+</sup> (beige mutation), CNcr.129P2-Tg<Tax53sm>Rcs (CD40 mutation), and B6.129-C<sub>80</sub>80<sub>190</sub>cr<sub>300</sub> (CD80/CD86 mutation) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Female SCID mice were purchased from Charles River Breeding Laboratories (Frederick, MD). Female C129(B6) mice on a BALB/c background were bred at our breeding facility at Biocon (Rockville, MD) from breeders obtained from Immunex (Seattle, WA). CD1 KO mice on a BALB/c background were bred under pathogen-free conditions from breeders obtained from Drs. W. E. Paul and M. Grusby, backcrossed eight times to a BALB/c background were bred at our breeding facility at Biocon (Rockville, MD) from breeders obtained from Immunex (Seattle, WA). CD1 KO mice on a BALB/c background were bred under pathogen-free conditions from breeders obtained from Drs. W. E. Paul and M. Grusby, backcrossed eight times to a BALB/c background were bred at our breeding facility at Biocon (Rockville, MD) from breeders obtained from Immunex (Seattle, WA). CD1 KO mice on a BALB/c background were bred under pathogen-free conditions from breeders obtained from Drs. W. E. Paul and M. Grusby, backcrossed eight times to a BALB/c background were bred at our breeding facility at Biocon (Rockville, MD) from breeders obtained from Immunex (Seattle, WA). CD1 KO mice on a BALB/c background were bred under pathogen-free conditions from breeders obtained from Drs. W. E. Paul and M. Grusby, backcrossed eight times to a BALB/c background were bred at our breeding facility at Biocon (Rockville, MD) from breeders obtained from Immunex (Seattle, WA). CD1 KO mice on a BALB/c background were bred under pathogen-free conditions from breeders obtained from Drs. W. E. Paul and M. Grusby, backcrossed eight times to a BALB/c background were bred at our breeding facility at Biocon (Rockville, MD) from breeders obtained from Immunex (Seattle, WA). CD1 KO mice on a BALB/c background were bred under pathogen-free conditions from breeders obtained from Drs. W. E. Paul and M. Grusby, backcrossed eight times to a BALB/c background were bred at our breeding facility at Biocon (Rockville, MD) from breeders obtained from Immunex (Seattle, WA). CD1 KO mice on a BALB/c background were bred under pathogen-free conditions from breeders obtained from Drs. W. E. Paul and M. Grusby, backcrossed eight times to a BALB/c background were bred at our breeding facility at Biocon (Rockville, MD) from breeders obtained from Immunex (Seattle, WA). CD1 KO mice on a BALB/c background were bred under pathogen-free conditions from breeders obtained from Drs. W. E. Paul and M. Grusby, backcrossed eight times to a BALB/c background were bred at our breeding facility at Biocon (Rockville, MD) from breeders obtained from Immunex (Seattle, WA). CD1 KO mice on a BALB/c background were bred under pathogen-free conditions from breeders obtained from Drs. W. E. Paul and M. Grusby, backcrossed eight times to a BALB/c background were bred at our breeding facility at Biocon (Rockville, MD) from breeders obtained from Immunex (Seattle, WA).

Generation of DCs

Bone marrow-derived DCs were generated according to a previously described procedure (8). Briefly, bone marrow cells were flushed from femurs of mice, filtered through a nylon mesh, depleted from erythrocytes with lysis buffer (BioWhittaker, Walkersville, MD) and plated out in bacteriological petri dishes (2 × 10<sup>5</sup> per 100-mm dish; 10 ml) in culture medium supplemented with mouse GM-CSF (20 ng/ml) and streptomycin (100 µg/ml), and 2-ME (50 µg/ml) at 37°C. The petri dishes were incubated overnight in culture medium supplemented with mouse GM-CSF (20 ng/ml) was added to the petri dishes. By comparison with the bone marrow-derived DCs, these splenic DCs were less mature by staining for the same markers. This phenotype is indicative of mature DCs. All experiments involving DCs, unless specified, staining splenic DCs, were performed with these bone marrow-derived DCs.

Freshly isolated splenocytes (10<sup>6</sup> cells/ml) were cultured for 4 days in culture medium containing mouse IL-2 (R&D Systems, McKinley Place, MN; 1000 U/ml). To remove cytokotoxicity mediated by mouse IL-2-activated T cells, cells were depleted of CD8+ T cells with mouse CD8 Dynal beads (Dynal Biotech, Lake Success, NY). Specific cytotoxic activities were determined in a standard 4-h 51Cr release assay at various E:T ratios. Briefly, graded doses of viable mouse IL-2-activated NK cells were plated in triplicate in 96-well U-bottom culture plates (Corning Glass, Corning, NY) and cocultured for 4 h with sodium chromate-labeled (100 µCi; NEN, Boston, MA) YAC-1 lymphoma cells (American Type Culture Collection). In some experiments, the CT26 tumor cell line was used as a target. Supernatants were collected, radioactivity measured, and specific lysis was calculated according to the equation: percentage of specific cytotoxicity = (experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm) × 100.

Maximum 51Cr release was determined from supernatants of lysed target cells incubated with Triton X-100 (5% v/v). Supernatant release was determined from target cells incubated without added effector cells.

Flow cytometry

Conventional mAb staining was done in PBS containing 0.01% sodium azide on ice. Cells were labeled with FITC- or PE-conjugated mAbs obtained from BD PharMingen (San Diego, CA). Purified anti-mouse CD16/CD32 mAb (BD PharMingen) was used to block Fc-mediated Ab binding. For each staining of interest, the appropriate isotype-matched control was included. All reagents were used at optimal concentration as determined experimentally. Flow cytometric analysis was performed with a FACSscan (BD Bioscience, Mountain View, CA). Data were collected on 10,000–10,000 viable cell events and analyzed with CellQuest software.

Immunohistochemistry

Mice were anesthetized with 0.2 ml i.p. Nembutal (12 mg/ml) and perfused intracardially with PBS and 4% paraformaldehyde (4% w/v) in PBS. The lungs were postfixed overnight and dehydrated with sucrose for 24 h (20% v/v). Sections were cut (20 µm) using a cryostat. Sections were stained free-floating for NK cells and CD8+ T cells using the avidin-biotin peroxidase procedure to be described. Tissues were preincubated in H<sub>2</sub>O<sub>2</sub> (3% v/v) to prevent nonspecific staining of endogenous peroxidases. Moreover, nonspecific binding of biotin-avidin system reagents was prevented by a commercial blocking kit (Vector Laboratories, Burlingame, CA). Sections were incubated overnight with appropriately diluted rabbit anti-asialo-GM1 (Wako, Richmond, VA) or purified rat anti-mouse CD8a (BD PharMingen) in BSA (1% w/v) and triton (0.3% v/v). Next, the tissues were incubated in an avidin-biotin complex solution (Vector Laboratories) and stained with a dianisobenzidine-peroxidase substrate kit (Vector Laboratories). Finally, sections were mounted on prefrsted slides using a gelatin solution, dehydrated and cover-slipped. As negative controls, the primary asialo-GM1 serum and CD8a Ab were replaced by normal rabbit serum (Intercell Technologies, Hopewell, NJ) and purified rat IgG2a Ab (BD PharMingen), respectively. The stained cells were counted for each section on three to five randomly chosen fields, each representing a total area of 0.6 mm<sup>2</sup>. Only peripheral lung tissue was examined and larger airways were excluded.

Statistical analysis

Statistically significant differences were evaluated with the nonparametric unpaired, two-tailed Mann-Whitney U test. Data were considered signifi- cantly different at p < 0.05.
Results

DC-mediated tumor protection in vivo

In an effort to generate rhabdomyosarcoma-specific CTLs, we immunized BALB/c mice with bone marrow-derived DCs pulsed with unique peptides (TAAs) spanning the tumor-specific translocation breakpoint regions. Indeed, after one immunization (i.v.) we were successfully able to isolate peptide-specific CTLs from the spleens of treated mice (data not shown). However, paradoxically and unexpectedly, in vivo protection against the growth of a challenge dose of CT26 tumor cells transfected with full-length human PAX3-FKHR cDNA (6) occurred in control mice inoculated with unpulsed DCs (Fig. 1a), making it impossible to evaluate the effect of immunization with DCs pulsed with specific peptide. In contrast, untreated and spleen cell-inoculated control mice experienced progressive tumor growth (Fig. 1, a and b). Not only was the inhibition of lung metastases nearly complete, but the DC-inoculated mice all survived, whereas control mice injected with this dose of CT26 tumor cells i.v. uniformly died within 2–3 wk (data not shown). This apparent peptide-unspecific tumor protection induced by unpulsed DC inoculation appeared to be systemic in nature, as evidenced by similar protections after different routes of DC administration (Fig. 1b). In particular, the fact that the DC could be injected s.c. indicates that the effect is not simply due to i.v. DC being filtered by the lung and affecting tumor growth locally. In addition, similar results were obtained with DCs generated in mouse serum, thus excluding the potential artifact of immunization against FCS-derived epitope, shared by DCs and tumor cells (data not shown).

Kinetic studies revealed that significant protection first occurred 4 days after DC inoculation (Fig. 1c). Furthermore, this tumor protection seemed to be a more general phenomenon not limited to CT26 tumor or BALB/c mice because inoculation of autologous unpulsed DCs also protected C57BL/6 mice against growth of LL/2 tumor cells (Fig. 1d).

NK cells are the effector cells in DC-mediated tumor protection in vivo

To investigate possible involvement of CD8+ T cells or NK cells as effectors in DC-mediated tumor protection, we treated mice with purified rat anti-mouse CD8 (2.43; Frederick Cancer Research and Development Center, Frederick, MD) and anti-asialo-GM1 (WAKO), respectively, before tumor challenge. Although CD8 depletion did not influence the protective efficacy of DC inoculation, depletion of NK cells decreased protection remarkably (Fig. 2a). Possible protection mediated by NK cells, known to be activated in a CD1 restricted fashion, was excluded because inoculation of CD1 KO DCs induced protection similar to that induced by wild-type DCs (Fig. 2a). Inoculation of DCs in Jh-KO mice (lacking mature B lymphocytes) induced comparable protection as in wild-type BALB/c mice, thus excluding possible requirement of B cells (Fig. 2b). To further verify the role of NK cells as effectors in tumor protection, DC inoculation was examined in beige mice (having a severe deficiency of NK cells), and did not lead to protection against tumor challenge (Fig. 2c), in contrast to control C57BL/6 mice in which DC inoculation protected (Fig. 5 and data not shown).

Interestingly, the spleens of DC inoculated mice displayed a significant increase in size and weight (127.2 ± 17.7 mg (±SD), in comparison with 86.2 ± 9.4 mg in untreated control mice; n = 10). In addition, IL-2-activated splenic NK cells from DC-inoculated mice exhibited a subset of NK cells with a distinctly different cell morphology (Fig. 3a) and showed significantly increased capacity to lyse NK-sensitive YAC-1 cells and CT26 tumor cells (Fig. 3, b and c), compared with control IL-2-activated NK cells, which under our culture conditions showed positive but modest killing activity. CT26 tumor cells do express MHC class I molecules (data not shown), thus indicating that class I deficiency was not the only mechanism of target cell discrimination by IL-2-activated NK cells.

Immunohistochemical analysis of lung sections revealed that in mice inoculated with DCs, NK cell numbers were significantly increased after CT26 tumor challenge. This increase in NK cell counts was most apparent after 3 days of tumor challenge (day 10 after DC inoculation), and gradually decreased to control levels after 10 days of CT26 administration (day 17 after DC inoculation) (Fig. 4, a and c), thus suggesting that at that time the mice had efficiently dealt with the tumor exposure. In contrast, CD8+ T cell numbers infiltrating the lung were not affected in mice inoculated with DCs and challenged with CT26 tumor cells (Fig. 4, b and d). Instead, these lungs showed CD8+ T cell numbers comparable with control levels as observed by others (10). Mice inoculated with DCs only displayed control levels of NK cell and CD8+ T cells (Fig. 4, e and d). In the untreated control mice, DC inoculated mice, and DC inoculated mice challenged with CT26 tumor, NK cells and CD8+ T cells were homogenously scattered in the lung tissue. No aggregates of NK cells and CD8+ T cells were found. At day 14 and 17 for mice injected with CT26 tumor only, the lung tissue was too much affected by pulmonary metastases to enable appropriate cell counting (data not shown).

DC-mediated tumor protection in vivo is dependent on expression of costimulatory molecules

DCs used in this study so far were obtained by in vitro culture of bone marrow precursors with GM-CSF and displayed a phenotype consistent with the mature myeloid stage with a high expression of CD80, CD86, MHC class I, and CD40 (see Materials and Methods; data not shown). To investigate the potential role of expression of costimulatory molecules on DCs in tumor protection in vivo, we inoculated wild-type mice with DCs generated from CD40 KO and CD80/CD86 KO mice. Although inoculation of CD40 KO DCs resulted in significantly reduced protective efficacy compared with wild-type DCs (Fig. 5a), CD80/CD86 KO DCs failed to generate significant protection (Fig. 5b). The importance of expression of costimulatory molecules on DCs was further supported by the absence of protection by immature freshly isolated splenic DCs (less bright for costimulatory molecules and MHC class I), and occurrence of protection after maturation of the DCs with murine CD40 ligand trimer (Immunex) (Fig. 5c).

To investigate whether release of NK cell-activating cytokines by inoculated DCs could account for tumor protection, we inoculated wild-type mice with DCs generated from IL-12 KO and IL-15 KO mice. However, because inoculation of IL-12 KO and IL-15 KO DCs induced protection similar to that induced by wild-type DCs (Fig. 5, d and e), production of these cytokines by DCs is not necessary for NK cell activation. In support of this conclusion, DC lysate failed to induce protection (data not shown).

DC inoculation provides long-term tumor protection

Remarkably, 14 mo after DC inoculation mice were still protected against tumor challenge. Protection was completely blocked by anti-asialo-GM1 treatment before CT26 administration, indicating that NK cells were still the effector cells. Depletion of CD8+ cells from mice with purified rat anti-mouse CD8 before tumor challenge did not affect protection by DCs (Fig. 6a). In addition, IL-2-activated splenic NK cells derived from mice inoculated with DCs 14 mo earlier showed a significantly enhanced YAC-1 lysis compared with those from mice not inoculated with DCs (Fig. 6b).
FIGURE 1. Inoculation of unpulsed autologous DCs protect against CT26 tumor challenge in BALB/c mice and against LL/2 tumor challenge in C57BL/6 mice. a, On day 0, BALB/c mice were inoculated (i.v.) with $10^6$ unpulsed DCs or left untreated. On day 7 all mice were challenged (i.v.) with $5 \times 10^5$ CT26 tumor cells and on day 19 the number of pulmonary metastases were evaluated. b, On day 0, BALB/c mice were inoculated (i.v., i.p., or s.c.) with $10^6$ unpulsed DCs or with $10^6$ unpulsed splenocytes (i.v.), or were left untreated. On day 7 all mice were challenged (i.v.) with $5 \times 10^5$ CT26 tumor cells, and on day 19 the number of pulmonary metastases was evaluated. c, On day 0, BALB/c mice were inoculated (i.v.) with $10^6$ unpulsed DCs. At different time points mice were challenged (i.v.) with $5 \times 10^5$ CT26 tumor cells and another 12 days later the number of pulmonary metastases was evaluated. d, On day 0, C57BL/6 mice were inoculated (i.v.) with $10^6$ unpulsed DCs or with $10^5$ unpulsed splenocytes (i.v.), or were left untreated. On day 7 all mice were challenged (i.v.) with $4 \times 10^5$ LL2 tumor cells and on day 28 the number of pulmonary metastases was evaluated. All data are representative of two to three separate experiments of at least five mice per treatment, with similar results.

FIGURE 2. NK cells are the effector cells in DC-mediated tumor protection in vivo. a, On day 0, BALB/c mice were inoculated (i.v.) with $10^6$ DCs, with $10^5$ splenocytes or with $10^5$ CD1 KO DCs, or were left untreated. Groups of DC inoculated mice received (i.p.) injections of either purified rat anti-mouse CD8 (500 μg, on days 5, 6, and 7), control purified rat IgG2b, anti-asialo-GM1 (50 μL, on days 4 and 7) or control normal rabbit serum, or were left untreated. On day 7 all mice were challenged (i.v.) with $5 \times 10^5$ CT26 tumor cells and on day 19 the number of pulmonary metastases was evaluated. b, On day 0, Jh-KO mice were inoculated (i.v.) with $10^6$ unpulsed DCs or were left untreated. On day 7 all mice were challenged (i.v.) with $5 \times 10^5$ CT26 tumor cells and on day 19 the number of pulmonary metastases was evaluated. c, On day 0, C57BL/6-KLHbg-J mice were inoculated (i.v.) with $10^6$ unpulsed DCs or were left untreated. On day 7 all mice were challenged (i.v.) with $4 \times 10^5$ LL2 tumor cells and on day 28 the number of pulmonary metastases was evaluated. All data are representative of two to three separate experiments of at least five mice per treatment, with similar results.
Furthermore, lung tissues of these mice showed significantly increased NK cell numbers after CT26 tumor challenge, whereas CD8+ T cell numbers were still at control levels (Fig. 6, c and d).

**DC-mediated tumor protection depends on interaction with CD4+ T cells**

Because NK cells are not supposed to have a memory function, we decided to study possible T cell involvement in DC-mediated NK cell activation. In accordance with this hypothesis, inoculation of DCs in SCID, Rag2 KO, and nude mice, all having normal NK cell numbers but lacking T cells, did not provide any protection against tumor challenge (Fig. 7a). To further investigate the T cell subset necessary for activating NK-mediated tumor protection, we depleted BALB/c mice of CD4+ T cells or CD8+ T cells before DC inoculation. In the case of anti-CD4 treatment, we treated the mice before DC inoculation to block any potential function of CD4+ T cells in the induction of NK cells, but we then waited 21 days before challenge with CT26 to allow CD4+ T cells to recover. This was done because other studies from our lab have found that depletion of CD4+ T cells immediately before tumor inoculation unmasks CD8+ T cell-mediated immunosurveillance that had been repressed by CD4+ suppressor cells in both this CT26 model (J. M. Park, M. Terabe, L. van den Broeke, D. Donaldson, and J. A. Berzofsky, manuscript in preparation) and a mouse fibrosarcoma (11). Whereas CD8 depletion did not affect the protective efficacy of inoculated DCs (Fig. 7b), CD4 depletion before DC inoculation completely blocked their protective efficacy (Fig. 7c), thus suggesting that intermediate activation of CD4+ T cells is essential for NK cell activation. This postulated role of CD4+ T cells upstream of NK cells in the mechanism of NK cell activation was further strengthened by findings that spleens of anti-asialo-GM1-treated mice did not show altered CD4+ T cell levels and that IL-2-activated splenic NK cells derived from CD4-depleted mice inoculated with DCs had reduced YAC-1 lysis (data not shown). An MHC-restricted mechanism of NK cell activation was further supported by findings that allogeneic DCs failed to protect against tumor challenge (data not shown).

**DC inoculation lacks therapeutic potential**

To investigate potential therapeutic benefit, we inoculated DCs at different time points after tumor challenge. However, even at distinctly reduced tumor doses, DC inoculation failed to cure established tumors, whereas IL-2-activated NK cells applied early after i.v. seeding of cancer cells reduced the metastatic tumor-cell growth only partially (Fig. 8).

**Discussion**

DCs have been frequently used for vaccination purposes. Successful immunizations have been reported with unfractionated tumor material (12), cell lysates (13, 14), apoptotic cells (15, 16), genetic material transferred by, e.g., tumor RNA/DNA (17, 18) or expressed in viral vectors (19), and fusions with whole tumor cells (20, 21). Yet, the most commonly used and clinically approved approach is loading empty MHC class I molecules with exogenous peptides (22–25). Indeed, we were successfully able to isolate peptide-specific CTLs specific for rhabdomyosarcoma after immunization with TAA pulsed DCs. However, regardless of the generation of TAA-specific CTLs, tumor protection in vivo by the induction of NK cells totally obscured any Ag-specific protection. Thus, the TAA-pulsed DC-induced antitumor immunity appeared to be mediated by stimulation of the innate as well as the adaptive immune system.

This phenomenon of DC induction of NK cell-mediated antitumor immunity was valid for the CT26 tumor model system (H-2d,b).
as well as for the LL/2 tumor model (H-2b), and occurred with different routes of DC inoculation. Thus, this DC-mediated protection is a systemic effect and seems to be independent of tumor source and mouse strain. Some evidence for related effects may have been seen in other systems as well, although the mechanisms were not worked out. For example, in a study of vaccination with tumor-lysate-pulsed DC in prevention of acute myelogenous leukemia, with or without bone marrow transplantation, the protective effect was found to be mediated primarily by CD4 and CD8 T cells, but some effect of NK cells was present based on depletion experiments (26). However, only a marginal effect was seen in mice immunized with unpulsed DC. Similarly, in a study of therapeutic vaccination with tumor extracts to treat B16 melanoma injected 6 days earlier, a delay in mortality was observed with unpulsed as well as pulsed DC; the protective effect was found to be mediated primarily by CD4 and CD8 T cells, but some effect of NK cells was present based on depletion experiments (27). The same group found some protection from liver metastases of an MCA-106 fibrosarcoma using unpulsed as well as pulsed DC; the protection by pulsed DC was found to be dependent on CD4+ T cells and CD8+ T cells, not NK cells, but the cells mediating protection by unpulsed DC were not examined (28). Thus, in the few prior examples in which some protective effect of unpulsed DC was observed, the cells involved were not defined or were not primarily NK cells, and the mechanism of induction of NK cells was not examined. In the present study, a necessary role for NK cells was initially evidenced by the markedly reduced protection after NK cell depletion with anti-asialo-GM1 before tumor challenge. Although the functional significance of the glycolipid determinant asialo-GM1 is unknown, most if not all, murine NK cells express this molecule. Hence, anti-asialo-GM1 is frequently used to deplete NK cells in vitro and in vivo. Yet, it is known that anti-asialo-GM1 is also expressed on other cells such as a subpopulation of T cells (29, 30). However, CD8+ T cell and NK T cell-mediated protection was excluded because CD8+ T cell depletion before tumor challenge did not influence the protective efficacy and CD11 KO DCs did protect equally well as wild-type DCs. In addition, DC inoculation in beige mice, lacking NK cells, failed to induce tumor protection. Furthermore, IL-2-activated splenic NK cells from DC inoculated mice displayed significantly higher YAC-1 and tumor lysis in comparison with those derived from control animals. Finally, immunohistochemical analysis of lung tissue revealed a DC-mediated influx of NK cells in tumor

FIGURE 4. Immunohistologically stained NK cells and CD8+ T cells in lung tissue of BALB/c mice. On day 0, BALB/c mice were inoculated (i.v.) with 10^6 unpulsed DCs or were left untreated. On day 7, each group was either challenged (i.v.) with 5 × 10^5 CT26 tumor cells or left unchallenged. On days 10, 14, and 17, groups of mice were taken for immunohistochemical examination of the lungs. Lung sections were stained for NK cells (a and c) or CD8+ T cells (b and d). Immunohistochemical staining (a and b), analysis of NK cell (c) and CD8+ T cell (d) numbers based on the immunohistochemical staining. +, Significant difference from counted cell numbers of control tissue, p < 0.001. All data are representative of two separate experiments of three to five mice per treatment, with similar results.
challenged mice, whereas CD8⁺ T cell numbers remained at control levels. It should also be noted that NKT cells do not express asialo-GM1, so the depletion studies with Abs to this marker, and the staining of cells in the lungs with these Abs, are consistent only with NK cells, not NKT cells.

Although DC-mediated T cell activation has been extensively documented, limited knowledge exists about the way DCs and the innate immune system are functionally coordinated to provide resistance against tumor challenge. Unexpectedly, DC-mediated NK cell activation appeared more likely to be the result of an intermediate interaction of DCs with CD4⁺ T cells rather than a direct effect of DCs on NK cells. This was evidenced by depletion of CD4⁺ T cells before DC inoculation, which yielded a lack of tumor protection and a lack of enhanced IL-2-activated NK cell activity. For these studies, we had to deplete the CD4⁺ T cells before DC inoculation, but then allow CD4⁺ T cells to recover before tumor inoculation, because other studies from our lab had shown that CD4⁺ cell depletion removed CD4⁺ NKT suppressive cells and unmasked CD8⁺ T cell-mediated immunosurveillance, thereby preventing growth of the CT26 cells (J. M. Park, M. Terabe, L. van den Broeke, D. Donaldson, and J. A. Berzofsky, manuscript in preparation) and other tumor cells (11). As NK cells do not require a thymus for their development, the absence of protection in athymic nude mice that lack T cells but have NK cells further indicates T cell involvement in DC-mediated tumor protection. In addition, DC inoculation failed to protect in SCID and Rag2 KO mice, lacking B and T cells but known to possess NK cells, whereas B cell KO mice still showed significant protection. In the case of nude, SCID, and Rag2 KO mice, the lack of CD4⁺ suppressor cells did not prevent tumor growth because these mice also lack the CD8⁺ effector cells mediating tumor immunosurveillance. Interestingly, tumor protection occurred earliest 4 days after DC inoculation, whereas direct NK cell activation usually arises within the first few hours (31). More strikingly, even though NK cells do not have memory, NK cell-mediated tumor protection was long lasting (14 mo). Also, allogeneic DCs did not induce this protection (data not shown), although they should be recognized by NK cells, also suggesting an intermediate cell between the inoculated DC and the NK effector cell. It is possible that CD4⁺ T cells must recognize self-MHC class II to form conjugates with the injected DC that last long enough to trigger whatever signal is involved, although it does not depend on a specific peptide in the MHC groove. In conclusion, our results indicate that besides the known role of CD4⁺ T cells in activation of CD8⁺ T cells (32, 33), CD4⁺ T cells are also capable to form a bridge between DCs and NK cells, and thus seem to play a central role in directing the immune system to either an adaptive or an innate immune response.

We do not know exactly how or where CD4⁺ T cells interact with NK cells. As we have seen similar NK-mediated protection by DC inoculation via i.v., s.c., or i.p. routes (Fig. 1b), the effect

![Graphs](http://www.jimmunol.org/DownloadedFrom/DC%20INDUCTION%20OF%20INNATE%20AND%20ADAPTIVE%20TUMOR%20IMMUNITY.png)
does not depend on the route of DC inoculation, and in particular does not depend on the DCs being trapped in the lung, which filters cells injected i.v. Also, the NK cells are detected in the spleen as well as in the lung, so it is likely that they are induced centrally in lymphoid tissue, and then disseminated to many sites. CD4\(^{+}\) T cells could directly activate NK cells, or could act through endogenous DCs or cytokines as mediators. NK cells are activated by both IL-2 and IL-15. The primary source of IL-2 is CD4\(^{+}\) T cells, so this could be at least part of the mechanism, and could be tested in IL-2 KO or receptor KO mice. Further, IL-15 is a critical cytokine for NK cell induction (34). Although we have shown in Fig. 5e that the inoculated DCs do not have to make IL-15, it is possible that the role of host CD4\(^{+}\) T cells is to activate host DCs to produce IL-15, via interaction of CD40 ligand on the T cell and CD40 on the DC.

With respect to the molecular mechanism of DC-mediated NK cell activation, the costimulatory molecules CD40 and CD80/CD86 appeared to play critical roles. Interaction of CD40 with CD40 ligand is an important costimulatory signaling pathway in the cross-talk between APCs and activated CD4\(^{+}\) Th cells. Originally, this receptor ligand system was mainly seen as a key component in the establishment of adaptive immunity, such as priming of T cells (35, 36) and the provision of CD4\(^{+}\) T cell help to B cells (37). Just recently, stimulatory anti-CD40 Ab was found to indirectly activate NK cells in vivo, and to generate tumor protection (38). Interestingly, direct interaction of CD40, expressed on, for example, DCs, with CD40 ligand on NK cells was not essential (38). Because NK cells express CD40 ligand but not CD40, the Ab could not have been acting directly on the NK cells, but was suggested to act on APCs to induce production of cytokines that then

**FIGURE 6.** NK cell-mediated tumor protection is long lasting. a, On day 0, BALB/c mice were inoculated (i.v.) with 10\(^6\) DCs or with 10\(^6\) splenocytes, or were left untreated. Fourteen months later, groups of DC inoculated mice received (i.p.) injections of either purified rat anti-mouse CD8 (500 \(\mu\)g, on three consecutive days), control rat IgG, anti-asialo-GM1 (50 \(\mu\)l, on two occasions with a 3-day interval) or control normal rabbit serum, or were left untreated. On the last day of Ab serum treatment, all mice were challenged (i.v.) with 5 \(\times\) \(10^5\) CT26 tumor cells and on day 19 the number of pulmonary metastases was evaluated. b, Graded numbers of IL-2-activated splenic NK cells from mice inoculated with DCs 14 mo earlier or from control uninjected mice were cocultured with \(^{51}\)Cr-labeled YAC-1 cells. After a 4-h incubation the specific lysis was evaluated. c and d. On day 0, BALB/c mice were inoculated (i.v.) with 10\(^6\) unpulsed DCs or were left untreated. Fourteen months later, each group was either challenged (i.v.) with 5 \(\times\) \(10^5\) CT26 tumor cells or left unchallenged. Another 3 days later, groups of mice were taken for immunohistochemical examination of the lungs. Lung sections were stained for NK cells (c) or CD8\(^{+}\) T cells (d). In c and d, immunohistochemical staining and cell counts based on these are shown. * Significant difference from control tissue, \(p < 0.01\).
activated the NK cells. This complicated scenario makes it difficult to use anti-CD40 to dissect the role of CD40/HLA class II helper cells in NK activation. The importance of CD40 activation in the generation of antitumor immunity is further emphasized by the inhibition of growth of tumors that are transduced with the CD40 ligand gene (39–42). CD40 ligand-expressing tumor cells seem to activate splenic DCs (39), and NK cells are found to play a prominent role in the primary rejection of tumor growth (39–42). The significance of costimulation between CD80/CD86 with CD28 in relation to activation of NK cells is less well described. However, direct CD80- and CD86-mediated NK cell activation has been demonstrated in in vitro model systems (43, 44). The importance of costimulation on the activation of NK cells is further demonstrated by studies with ICAM and CD70 (45, 46).

Activation of DCs through the CD40-CD40 ligand interaction with T cells can lead to the production of soluble mediators, such as IL-12, IL-15, and IL-18, which may activate NK cells (34, 47). However, our results using DCs unable to make IL-12 or IL-15 indicate that endogenous release of IL-12 and IL-15 derived from the inoculated DCs themselves is not required for NK cell activation, so provision of these cytokines by the DC inoculum is not the mechanism. DC production of IL-12, while able to enhance CTL induction, has similarly been shown not to be essential for induction of CD8+ CTL either (48). In addition, the absence of protection by DC lysate renders it less likely that the inoculated DC are merely a source of cytokine. However, a more indirect mechanism, such as NK cell activation mediated by cytokines released from cells of the recipient mice in response to the DC inoculation cannot be ruled out. This possibility would be harder to test because using hosts deficient in a cytokine like IL-15 may make it difficult to induce NK cells, as IL-15 is necessary for such cells to develop (34), but such a result would not prove that IL-15 was involved in the mechanism of the DC inoculation effect.

In contrast to protection, DC inoculation was consistently insufficient to generate therapeutic benefit for CT26 bearing mice, whereas IL-2-activated NK cells induced only partial protection. However, it should be realized that it has been harder to achieve

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**FIGURE 7.** CD4+ T cells play an intermediate role in DC-mediated NK cell activation. *a*. On day 0, BALB/c, Rag2 KO, SCID, and nude mice were inoculated (i.v.) with 10^6 unpulsed DCs (i.v.), or were left untreated. On day 7 all mice were challenged (i.v.) with 5 × 10^5 CT26 tumor cells and on day 19 the number of pulmonary metastases was evaluated. *b*. On three consecutive days, BALB/c mice received (i.p.) injections of purified rat anti-mouse CD8 (500 μg), or were left untreated. On the last day of Ab treatment, each group was inoculated (i.v.) with 10^6 unpulsed DCs (i.v.), or were left untreated. Seven days later, all mice were challenged (i.v.) with 5 × 10^5 CT26 tumor cells and another 12 days later the number of pulmonary metastases was evaluated. *c*. On three consecutive days, BALB/c mice received (i.p.) injections of purified rat anti-mouse CD4 (500 μg), or left untreated. On the last day of Ab treatment, each group was inoculated (i.v.) with 10^6 unpulsed DCs (i.v.), or were left untreated. Twenty one days later, all mice were challenged (i.v.) with 5 × 10^5 CT26 tumor cells and another 12 days later the number of pulmonary metastases was evaluated.

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**FIGURE 8.** DC inoculation lacks therapeutic potential. On day 0, BALB/c mice were challenged (i.v.) with 5 × 10^4 CT26 tumor cells. On days 1, 2, and 3, groups of mice were inoculated (i.v.) with either 10^6 unpulsed DCs or 10^6 unpulsed IL-2-activated NK cells, or were left untreated. On day 22 the number of pulmonary metastases was evaluated.
vaccine-induced regression of pre-established tumors than prevention of tumors. One of the possible reasons for the disappointing therapeutic outcome is that immune cells may have become defective in the tumor bearing mice. Tumor cells themselves or tumor infiltrating immunomodulatory cells can release immunosuppressive mediators such as TGF-β, IL-10, and PGE₂, that inactivate host T cells or NK cells (49). For example IL-13, a potent stimulator and activator of TGF-β in vivo (50), released by CD4⁺ NKT cells can down-regulate tumor immunosurveillance (11). In this respect, naive human CD4⁺ T cells, when exposed to TGF-β, become hyporesponsive to DC stimulation (51). Because inoculated CT26 tumor cells failed to grow in either CD4⁺ T depleted mice or CD1 KO mice (lacking CD1-restricted NKT cells), because in the absence of these suppressive cells, CD8⁺ T cells arise that mediate immunosurveillance and reject the tumor (J. M. Park, M. Terabe, L. van den Broeke, D. Donaldson, and J. A. Berzofsky, manuscript in preparation), the lack of therapeutic benefit of DC treatment was possibly mediated by tumor-induced IL-13 production, which has been shown to be a mediator of the negative regulation of immunosurveillance (11) (J. M. Park, M. Terabe, L. van den Broeke, D. Donaldson, and J. A. Berzofsky, manuscript in preparation). In contrast to systemic application of DCs, the therapeutic potential of intratumoral injected DCs, via induction of Ag-specific CD8⁺ T cells, has been more specifically documented (52, 53).

The DCs generally used in this study were derived from bone marrow and displayed a morphology and phenotype conforming to mature CD11c⁺, CD40⁺, CD80⁺ myeloid DCs. However, it should be noted that the concept of a stable DC phenotype over-simplifies their complex biology. In the immature stage of differentiation, DCs express low levels of costimulatory molecules and are functionally specialized in capturing and processing protein Ags. Mature DCs express elevated levels of MHC and costimulatory molecules, and display a high efficiency in stimulating T cells, accompanied with down-regulated Ag uptake and processing mechanisms (54). Murine DC subsets can display a strong functional heterogeneity (9, 55–59), and may be influenced by cytokines released in the microenvironment. The importance of understanding the nuances of DC phenotypes in vaccination protocols is stressed by our finding that, in contrast to CD40 ligand-induced matured splenic DCs, freshly isolated immature splenic DCs failed to reduce the number of pulmonary metastases after CT26 challenge.

In conclusion, the value of DC immunization as an approach to design tumor vaccines can lead to the induction of CTL-mediated as well NK cell-mediated antitumor immunity. CD4⁺ T cells play a central role in promoting the direction of the immune response and hence form a bridge between the adaptive and innate immune system. The novel role for CD4⁺ T cells in activating NK cells demonstrates a new mechanism by which CD4⁺ T cells regulate innate immunity. The importance of DC-based vaccinations is further stressed by the high polymorphism of the human HLA system that complicates identifying and applying TAAAs and consequently complicates stimulation of adaptive immune responses. In addition, many tumors escape from CTL recognition because of down-regulated MHC class I expression, whereas this down-regulation releases the NK cells from inhibitory signals and make tumors susceptible to NK-mediated cytolysis. Although, the DC-mediated induction of NK cells that clear tumor has so far been successful only in protection, not therapy, it may be valuable in the prevention of metastases after surgical resection of the primary tumor, as the CT26 tumor model used here is designed to mimic pulmonary metastasis of a colon carcinoma.

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References


