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NK and CD4 Cells Collaborate to Protect against Melanoma Tumor Formation in the Brain

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NK cells represent a potent immune effector cell type that have the ability to recognize and lyse tumors. However, the existence and function of NK cells in the traditionally “immune-privileged” CNS is controversial. Furthermore, the cellular interactions involved in NK cell anti-CNS tumor immunity are even less well understood. We administered non-Ag-loaded, immature dendritic cells (DC) to CD8 knockout (KO) mice and studied their anti-CNS tumor immune responses. DC administration induced dramatic antitumor immune protection in CD8 KO mice that were challenged with B16 melanoma both s.c. and in the brain. The CNS antitumor immunity was dependent on both CD4⁺ T cells and NK cells. Administration of non-Ag-loaded, immature DC resulted in significant CD4⁺ T cell and NK cell expansion in the draining lymph nodes at 6 days postvaccination, which persisted for 2 wk. Finally, DC administration in CD8 KO mice was associated with robust infiltration of CD4⁺ T cells and NK cells into the brain tumor parenchyma. These results represent the first demonstration of a potent innate antitumor immune response against CNS tumors in the absence of toxicity. Thus, non-Ag-loaded, immature DC administration, in the setting of CD8 genetically deficient mice, can induce dramatic antitumor immune responses within the CNS that surpass the effects observed in wild-type mice. Our results suggest that a better understanding of the cross-talk between DC and innate immune cells may provide improved methods to vaccinate patients with tumors located both systematically and within the CNS. The Journal of Immunology, 2006, 177: 8448–8455.
activate NK cells and induce protective antitumor immunity to s.c. tumors in CD8α knockout (KO) mice. In this study, we demonstrated that non-Ag-loaded, immature DC could induce robust antitumor immunity to i.c. tumors in CD8α/KO mice that was dependent upon CD4+ T cells and NK cells. We also documented the ability of non-Ag-loaded, immature DC to induce the selective expansion of CD4+ T cells and NK cells in the lymph nodes draining the DC injection site, as well as the infiltration into the CNS tumor parenchyma, which are findings not previously reported. A better understanding of the mechanisms underlying the ability of DC to activate NK cells in this model should lead to a more fundamental understanding for similar actions in wild-type mice. Together with our ability to serially monitor tumor growth in vivo, we believe that these studies contribute important new information about the cellular mechanisms of antitumor immunity. These results suggest to us that DC-based strategies designed to activate NK cells could synergize with other Ag-specific DC vaccines for the treatment of tumors located within the CNS.

**Materials and Methods**

### Animals and cell lines

C57BL/6, CD8 α-chain KO mice (CD8α KO, B6.129S2-Cd8αtm1Mak, backcrossed over 28 generations) were purchased from The Jackson Laboratory and were bred and kept under defined-flora pathogen-free conditions at the American Association of Laboratory Animal Care-approved Animal Facility of the Division of Experimental Radiation Oncology, University of California. Mice were handled in accordance with the University of California animal care policy. The B16 murine melanoma cell line was obtained from the American Type Culture Collection and maintained in DMEM with 10% FCS, penicillin/streptomycin and l-glutamine. B16 cells stably expressing firefly luciferase (B16-Fluc) were created as described elsewhere (20). Growth rates of B16-Fluc both in vitro and in vivo were similar to those of parental B16 cells.

### Bone marrow-derived DC

The development of DC from murine bone marrow progenitor cells was performed as previously published (21–23). Bone marrow cells were cultured overnight in RPMI 1640 (Invitrogen Life Technologies) with 10% FCS (Gemini Bio-Products) and 1% (v/v) penicillin, streptomycin, and Fungizone (Gemini Bio-Products) in a petri dish. Nonadherent cells were replated on day 1 at 2–3 × 10^6 cells/well in 24-well plates with murine GM-CSF (100 ng/ml; Amgen) and murine IL-4 (500 U/ml; R&D Systems). Cultures were maintained in RPMI 1640 medium containing 10% FCS and antibiotics (complete medium) at 37°C in 5% CO2. On day 4, 80–90% of the medium was removed, and adherent cells were refed with an addition of 1 ml per well of complete medium plus cytokines, DC were harvested as the loosely adherent cells from the day-8 cultures. DC were resuspended at 2–5 × 10^6 cells/ml in serum-free RPMI 1640 medium, and 1 × 10^6 unpulsed, immature DC were injected s.c. per mouse.

### In vivo mAb treatment

In vivo mAb ablation of CD4+ (clone GK1.5; Bio Express) T cell subsets and NK1.1+ (clone PK136; Bio Express) cells was performed by i.p. injection of 100 μg of purified endotoxin-free mAb/mouse/injection on days −5, −3, and −1 before tumor challenge, and every 6 days thereafter. Irrelevant CD8α mAb (clone 2.43; Bio Express) was given to CD8α KO mice in selected experiments to confirm that the injection of irrelevant mAbs did not nonspecifically influence the experimental paradigm. Monitoring of successful depletion was performed by flow cytometry on splenocytes harvested on the day of tumor inoculation for CD4-depleting...
mAb. NK depletion was confirmed by NK cell activity microcytotoxicity assays using the NK-sensitive Yac-1 cell line as target cells (Yac-1 assay).

Animal studies and experimental paradigm
Mice were s.c. injected with 5 × 10³–1 × 10⁶ non-Ag-loaded, immature DC on the right flank. The mice were then challenged s.c. or in the brain (i.c.) 1–2 wk later with B16-Fluc cells (s.c., 1 × 10⁵ cells; i.c. 1 × 10⁴ cells). Before tumor challenge, B16-Fluc cells were grown in supplemented DMEM, trypsinized with 0.05% trypsin-EDTA (Invitrogen Life Technologies), enumerated using a hemacytometer, and then washed three times in Dulbecco’s PBS (Invitrogen Life Technologies). For the i.c. implantation of B16-Fluc melanoma cells, animals were first anesthetized with ketamine/xylazine. The head was shaved and the skull exposed. Thereafter, the animal was positioned into a stereotaxic frame (David Kopf Instruments) with small animal earbars. A burr hole was made using a Dremel drill ~1.5-mm lateral and 1-mm posterior from the intersection of the coronal and sagittal sutures (bregma). A total of 1 × 10⁴ cells was injected using a Hamilton syringe at a depth of 3 mm in a volume of 2 μl. The s.c. B16-Fluc tumor growth was induced similarly by first anesthetizing and shaving the mice, followed by the injection of 1 × 10⁴ tumor cells under the skin.

Cytotoxicity assays
Splenocytes were harvested 10–14 days after DC administration, depleted of RBC by hypotonic lysis, cultured in vitro (without stimulator cells) for 96 h in the presence of 10 U/ml IL-2 (Chiron), and assayed in a standard 4-h ⁵¹Cr release test (10, 24). NK depletion by clone PK136 was achieved by three i.p. injections of 200 μg of purified Ab (Bio Express). Successful depletion was assessed by testing the ability to lyse chromated Yac-1 cells by IL-2-stimulated splenocytes (Yac-1 assay). Cells were then washed and added to effector cells in the microcytotoxicity essay plates.

FACS analysis
Spleens and lymph nodes were harvested from CD8α KO mice that received control Ag-unloaded DC administration, and a single cell sus-

In vivo fluorescence imaging
In vivo, bioluminescent imaging (BLI) was performed on tumor-bearing mice. Before imaging, mice were anesthetized with a mixture of ketamine/xylazine. The head was shaved and the skull exposed. Thereafter, the animal was positioned into a stereotaxic frame (David Kopf Instruments) with small animal earbars. A burr hole was made using a Dremel drill ~1.5-mm lateral and 1-mm posterior from the intersection of the coronal and sagittal sutures (bregma). A total of 1 × 10⁴ cells was injected using a Hamilton syringe at a depth of 3 mm in a volume of 2 μl. The s.c. B16-Fluc tumor growth was induced similarly by first anesthetizing and shaving the mice, followed by the injection of 1 × 10⁴ tumor cells under the skin.

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were then incubated with primary Abs to CD3ε (500A2; BD Pharmin-gen), CD4 (RM4-5; BD Pharmingen), and Ly49GH (4D11; BD Biosciences). The primary mAb incubation step was followed by a biotin-ylated secondary mAb (Vector Laboratories) and developed with a diaminobenzidine substrate kit (Vector Laboratories). Negative controls consisted of isotype-matched rat or hamster IgG in lieu of the primary mAbs listed above.

Statistical analysis
All error bars represent SEM. Continuous variables were compared using a paired Student’s t test. Significant differences in BLI data were obtained using the ANOVA Repeated Measures function for groups of mice over the same experimental time course. The survival curves were determined using the Kaplan-Meier method. The log-rank test was used to compare survival between study and control groups. Values for p are two-tailed, and p < 0.05 was considered statistically significant. Graphs were constructed using Sigma Plot (Systat Software) and statistical functions were analyzed using Systat 11 software.

Results
Antitumor protection against s.c. and CNS melanomas after DC administration in mice deficient in CD8α cells
We recently demonstrated that non-Ag-loaded, immature DC administration to CD8α KO mice generated robust Ag nonspecific antitumor immune responses to an otherwise lethal s.c. B16 melanoma challenge (12). However, the question of whether DC administration could induce antitumor immunity to tumors located within “immune privileged” sites, such as the brain, remains unresolved. To answer this question, we administered non-Ag-loaded, immature DC once to groups of CD8α KO mice and then challenged them with lethal doses of B16-Fluc melanoma cells s.c. or in the brain. B16-Fluc melanomas express firefly luciferase, which enables accurate, real-time visualization of tumor
progression (20, 26–28). In this study, B16-Fluc cells were grown in culture medium before tumor implantation. This model contrasts with our previous study, which used in vivo passaged, single cell tumor digests (12). However, because both the tumor growth kinetics and survival were very similar in both experimental studies, we do not believe this alteration has influenced the experimental model. Using this newly developed tumor model, we confirmed our earlier findings with complete s.c. tumor protection using in vivo BLI (Fig. 1). These studies also demonstrated that non-Ag-loaded, immature DC administration could generate significant antitumor protection to an i.c. challenge in CD8 KO mice, which often resulted in long-term (>60 days) survival in ~20% of mice (Fig. 2). Non-Ag-loaded, immature DC administration, however, did not generate significant protection to an i.c. B16-Fluc melanoma challenge in wild-type C57BL/6 mice (data not shown). The antitumor immunity induced in CD8 KO mice did surpass that induced in wild-type C57BL/6 mice vaccinated with tumor Ag-pulsed DC (21–23, 29). Collectively, these studies demonstrated that antitumor immunity could be generated to both s.c. and i.c. B16-Fluc melanoma and could be monitored using BLI.

CD4+ and NK1.1+ cells are required for CNS antitumor immunity that is induced by DC administration

To examine the role of lymphocyte subsets in this model, we performed in vivo mAb depletion of CD4+ and NK1.1+ cells before DC administration and i.c. B16-Fluc melanoma challenge. In vivo depletion of either CD4+ cells or NK1.1+ cells significantly reduced the survival benefit induced by DC administration (Fig. 3C and data not shown). We believe that these cell types were indeed critical to the antitumor immunity induced by non-Ag-loaded, immature DC administration. In the absence of DC administration, CD4 and NK mAb depletion did not alter the survival of CD8 KO mice to an i.c. B16-Fluc melanoma challenge (data not shown). Furthermore, BLI visualization of i.c. B16-Fluc tumor progression demonstrated significantly reduced i.c. tumor burden in DC-sensitized mice compared with DC-sensitized mice that were depleted of CD4+ cells, NK1.1+ cells, or placebo-treated control CD8α KO mice (Fig. 3, A and B). These results strongly suggest that both CD4+ cells and NK1.1+ cells are critical immune cell subsets necessary for the anti-CNS tumor immunity generated by DC administration in CD8α KO mice.
DC administration induces an early increase of CD4+ and NK1.1+ lymphocytes in the draining lymph nodes that express activation markers and CD62 ligand (CD62L)

Based on these results, we were interested to understand the underlying mechanisms of how non-Ag-loaded, immature DC administration induced anti-CNS tumor immunity in CD8α KO mice. To answer this concern, we performed ex vivo FACS analyses on spleen and lymph node cells to test whether DC administration induced selective expansion and activation of CD4+ T cells and NK cells in a non-tumor-bearing state. As shown in Fig. 4A, DC administration induced a rapid expansion of CD4+ T cells (CD3ε+CD4+) and NK cells (CD3ε−NK1.1+) in the lymph nodes draining the DC s.c. injection site. The increased draining lymph node cellularity peaked at 5–7 days, and remained elevated to at least 2 wk postvaccination (Fig. 4A). FACS analysis of lymph node cells draining the DC injection site revealed CD3ε− CD4+ T cell and CD3ε−NK1.1+ populations that expressed low levels of CD62L (L-selectin) at 2 days (Fig. 4B and data not shown). This same population also expressed the early activation marker, CD69, and was CD44-positive (Fig. 4C). By day 7 post-DC injection, CD4+CD62L− T cells were present in the spleen (data not shown). Therefore, non-Ag-loaded, immature DC administration induces a regional increase in the absolute number, as well as activation status, of NK cells and CD4+ T cells draining the injection site.

NK cells from DC-sensitized CD8α KO mice have cytotoxic activity in vitro

To test what types of immune cells were likely responsible for the in vivo antitumor activity, we analyzed the lytic activity of splenocytes from groups of mice that received non-Ag-loaded, immature DC administration or a placebo control. Splenocytes from DC-sensitized CD8α KO mice exhibited high levels of lytic activity against B16 melanoma cells in ex vivo microcytotoxicity assays (Fig. 5). However, The lytic activity was not observed in NK-depleted CD8α KO mice (Fig. 5). Therefore, the predominant cytotoxic cells in the CD8α KO system have a NK phenotype and activity.

DC administration induces trafficking of CD4+ T cells and NK cells into CNS tumors

Finally, we sought to determine the effector cells responsible for decreased tumor burden and increased survival associated with DC administration. Groups of CD8α KO mice were treated as before, implanted with B16 melanoma cells in the brain, and then sacrificed at defined time points for immunohistochemical staining of tumor-associated lymphocytes. Histochemical staining (H&E) demonstrated smaller i.c. tumor burden in DC-sensitized mice, which corroborated our previous survival and BLI data (Fig. 6). At 7 days postimplantation, Ly49G+–immunoreactive NK cells were readily seen around the tumor and infiltrating the i.c. tumor pock-ets in mice injected with non-Ag-loaded, immature DC compared with mice that received only Dulbecco’s PBS injection (Fig. 6). Qualitatively, Ly49G+ NK cells declined at the tumor site by 14 days, but were still visualized. Large numbers of CD4+ cells were observed both at day 7 and day 14 in DC-sensitized mice, but were almost completely absent in control-treated mice. In general, we
observed an early influx of NK cells infiltrating the tumor parenchyma, whereas enhanced tumor infiltration of CD4+ cells was consistently observed throughout the time period. FACs analysis of tumor-infiltrating lymphocyte populations suggests that these cells are predominantly CD3+CD4+ T cells and CD3+NK1.1+ (data not shown). These results collectively suggest that the anti-tumor response induced by DC administration is associated with CD4+ T lymphocytes and NK cells in and around the i.c. tumor, which directly correlates with decreased i.c. tumor burden.

**Discussion**

The studies outlined in this study were performed to model an immunotherapeutic strategy targeting melanomas in the “immunopriviliged” CNS, as well as to shed light on the important cellular interactions with DC, NK cells, and CD4+ T cells. These results built upon our previous work documenting the ability of DC to consistently observed throughout the time period. FACS analysis which directly correlates with decreased i.c. tumor burden. The studies outlined in this study were performed to model an otherwise lethal challenge of B16 melanoma cells in the brain. This antitumor immunity was even more effective than our previous experience with TAA peptide-loaded DC vaccination (21–23, 24). The privileged nature of the CNS, as well as to shed light on the important cellular interactions with DC, NK cells, and CD4+ T cells. These results built upon our previous work documenting the ability of DC to consistently observed throughout the time period. FACS analysis which directly correlates with decreased i.c. tumor burden.

**References**


