

Peptide-pulsed Dendritic Cells Induce Antigen-specific, CTL-mediated Protective Tumor Immunity

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Summary

Cytotoxic T lymphocytes (CTLs) are a critical component of the immune response to tumors. Tumor-derived peptide antigens targeted by CTLs are being defined for several human tumors and are potential immunogens for the induction of specific antitumor immunity. Dendritic cells (DC) are potent antigen-presenting cells (APCs) capable of priming CTL responses in vivo. Here we show that major histocompatibility complex class I-presented peptide antigen pulsed onto dendritic APCs induces protective immunity to lethal challenge by a tumor transfected with the antigen gene. The immunity is antigen specific, requiring expression of the antigen gene by the tumor target, and is eliminated by in vivo depletion of CD8⁺ T cells. Furthermore, mice that have rejected the transfected tumor are protected from subsequent challenge with the untransfected parent tumor. These results suggest that immunization strategies using antigen-pulsed DC may be useful for inducing tumor-specific immune responses.

CTLs are a critical component of host immunity to tumors (1–3). CTLs typically recognize peptides derived from endogenously synthesized proteins and presented by MHC class I molecules on the surface of target cells (4, 5). Peptides recognized by tumor-specific CTLs are being defined for several human tumors and MHC class I alleles (6–8). Though the recognition of peptide–class I complexes is sufficient to trigger target cell lysis, priming of CTL responses requires the presentation of the relevant antigen by professional APCs capable of providing “costimulation.” Dendritic cells (DC) are efficient APCs for CTL induction and may be an attractive adjuvant for immunizations using peptide tumor antigens (9–13). Recent advances have made it feasible to acquire significant numbers of DC from a variety of sources, including human peripheral blood (13, 14) and murine bone marrow (15–17). Furthermore, cell surface class I molecules on DC are accessible to exogenously added peptides in vitro. DC pulsed with peptides in vitro present functional peptide–class I complexes on the cell surface and can stimulate antigen-specific CTL when administered in vivo (18–23). Here we show that DC pulsed with peptide antigen in vitro and administered to animals s.c. induce antigen-specific, CTL-mediated protection against a lethal tumor challenge.

Materials and Methods

Mice and Cell Lines. Female C57BL/6 mice, 5–8 wk old, were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed at the Central Animal Facility of the University of

Pittsburgh. EL4 is a C57BL/6 T thymoma and EG7 is a chicken egg OVA-expressing subclone of EL4 (24). B16, the C57BL/6-derived melanoma, was obtained from the American Type Culture Collection (ATCC, Rockville, MD). MO5 was constructed by transfection of B16 with the pAc-neo-OVA plasmid as previously described (24, 25).

Antigen and Antibodies. The peptide corresponding to the amino acid sequence of OVA residues 257–264 (SIINFEKL) (26, 27) (K^b restricted) was synthesized by the Peptide Synthesis Facility of the University of Pittsburgh Medical Center. mAbs were prepared from the hybridomas GK1.5 (anti-CD4, ATCC TIB-207), 2.43 (anti-CD8, ATCC TIB-210) or 30-H12 (anti-Thy 1.2, ATCC TIB-107). Ascites containing anti-CD8 antibodies were raised in BALB/c nu/nu mice by i.p. injection of GK1.5 cells (3×10^6) and IFA (0.5 ml/mouse).

Preparation of DC. DC were prepared from bone marrow by described techniques (15). Briefly, bone marrow cells were depleted of lymphocytes and cultured overnight in RPMI-1640 supplemented with 10% FCS, L-glutamine, antibiotics, and 2-ME in 24-well plates at 10^6 cells/well. Cells were replated on day 1 at 2.5×10^5 cells/well with GM-CSF (10^3 U/ml; Sigma Chemical Co., St. Louis, MO) and murine rIL-4 (10^3 U/ml; Genzyme Corp., Cambridge, MA), and loosely adherent cells were harvested on day 8. By flow cytometric analysis, these DC expressed CD45, CD44, CD11b (Mac-1), CD18, CD80, CD86, and class I and class II MHC antigens (data not shown). DC were pulsed for 2 h at 37°C with or without OVA peptide (20 ng/ml) + β_2 -microglobulin ($[\beta_2M]$ 10 μ g/ml, human; Sigma Chemical Co.) (28) in reduced serum media (Optimem; Gibco Laboratories, Grand Island, NY). Cells were then washed extensively, resuspended in PBS, and irradiated (2,000 rad) before injection into naive mice.

Protection Assays. C57BL/6 mice were immunized s.c. in both lower flanks with either peptide-pulsed DC or nonpulsed

DC ($3 \times 10^4/100 \mu\text{l}/\text{side}$), peptide + $\beta 2\text{M}$, or PBS on day 0 and boosted on day 7. 7–10 d later, mice were challenged by intradermal (i.d.) injection in the midflanks bilaterally with MO5 or B16 ($2.5 \times 10^4/100 \mu\text{l}/\text{side}$) in PBS. Injected cells were $>95\%$ viable as determined by trypan blue exclusion. In some experiments, in vivo CD8 depletion was carried out by i.p. injection of ascites (1 mg/ml, $200 \mu\text{l}/\text{mouse}$) into mice on days 7 and 9 after the final immunization. The size of the tumors was assessed three times weekly and recorded as tumor area (mm^2) by measuring the largest perpendicular diameters. Data are reported as the average tumor area \pm SEM. Survival is recorded as the percentage of surviving animals. Mice were killed in accordance with established guidelines. All experiments included five mice per group and were repeated at least three times. In some experiments, mice surviving an initial MO5 challenge were rechallenged (along with naive control mice) with the parent B16 ($2.5 \times 10^4/100 \mu\text{l}/\text{side}$ in PBS).

Cytotoxicity Assays. Splenocytes (30×10^6) harvested from mice 7–10 d after the last immunization were restimulated by coculture with mitomycin C-treated EG7 cells (7.5×10^6) and effector cells harvested 5 d later. Cytotoxicity assays were performed as described (28) with minor modifications. Briefly, target cells were labeled by incubation in RPMI (10% FCS) with ^{51}Cr ($100 \mu\text{Ci}$; NEN, Boston, MA) for 18 h at 37°C , washed extensively, and cocultured at 2×10^4 cells/well with effector cells (at the ratios given in the figures) in 96-well round-bottom plates ($200 \mu\text{l}/\text{well}$) for 4 h at 37°C . In some cases the effector cells were depleted of T cell subsets using mAb plus complement before assay as described (29). $100 \mu\text{l}$ of supernatants from triplicate cultures was collected and counted. Data points are expressed as the mean percent specific release of ^{51}Cr from target cells and were calculated as described (27).

Results and Discussion

To determine the capacity of peptide-pulsed DC to induce protective immunity, we used a tumor model based on the poorly immunogenic C57BL/6 mouse-derived melanoma B16 and the OVA-transfected B16 subclone MO5. MO5 endogenously synthesizes OVA and generates and presents the OVA peptide SIINFEKL with its surface class I molecule K^b (25). The expression of the OVA antigen does not significantly increase the immunogenicity of this tumor in vivo as tumor growth and progression is similar to that of the untransfected parent tumor (25).

In initial experiments, we evaluated the capacity of OVA peptide-pulsed DC to induce CTLs capable of lysing the OVA-expressing melanoma in vitro. Bone marrow-derived DC (15) were prepulsed with SIINFEKL in the presence of exogenously added $\beta 2\text{M}$ and washed extensively. Peptide-pulsed DC specifically stimulated the SIINFEKL + K^b -specific T-T hybridoma RF33.70 (27), indicating the presence of functional SIINFEKL + K^b complexes on the DC surface (data not shown). The peptide-pulsed DC were irradiated and injected s.c. into naive mice. Immunized mice were boosted 7 d later. In vitro restimulated spleen cells from these mice lysed the OVA transfectant MO5, but not the untransfected parent melanoma B16 (Fig. 1 A). Similarly, these effector cells lysed the OVA-expressing murine thymoma EG7 (Fig. 1 B), but not the untransfected parent

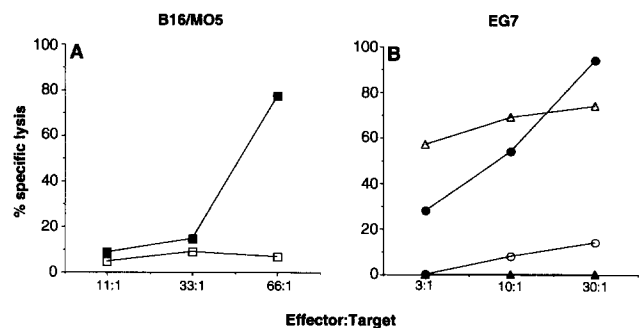


Figure 1. Immunization with OVA peptide-pulsed DC induces antigen-specific CD8⁺ effector cells that lyse OVA-expressing tumor targets. (A) C57BL/6 mice were immunized s.c. in the lower flanks with peptide-pulsed DC or PBS (not shown) on days 0 and 7. Splenocytes taken 7 d after the last immunization were restimulated in vitro for 5 d with mitomycin C-treated OVA-expressing EG7 cells and assayed for cytolytic function against ^{51}Cr -labeled B16 melanoma (open squares) or MO5 (solid squares). (B) Effector populations were incubated with complement alone (solid circles) or with mAbs against CD4⁺ (open triangles), CD8⁺ (open circles), or Thy 1.2⁺ (solid triangles) lymphocytes and complement, then assayed for cytolytic activity against EG7 cells. Specific release for EL4 targets in B was 5, 3, and 0% at E/T ratios of 30, 10, and 3:1.

tumor EL4 (Fig. 1). Thus tumor cell lysis was antigen specific, depending on expression of OVA by the tumor target. Depletion of T cell subsets from effector populations using mAbs demonstrated that lysis depended on Thy 1⁺CD8⁺ subsets characteristic of MHC class I-restricted CTL effector cells (Fig. 1 B). These results are in agreement with those recently reported by Porgador and Gilboa (23) demonstrating CTL priming after i.v. administration of peptide-pulsed DC.

To determine the capacity of peptide-pulsed DC to induce protective tumor immunity, groups of mice were subcutaneously immunized with SIINFEKL-pulsed DC, boosted 7 d later, and then challenged i.d. at a distant site with the MO5 melanoma. Immunized mice were protected from tumor growth locally (Fig. 2 A) and from death (Fig. 2 C). Tumors in control mice (PBS immunized) grew progressively (Fig. 2 A) and were lethal (Fig. 2 C). Mice immunized with DC not pulsed with SIINFEKL were not protected (Fig. 2, B and D), suggesting that subcutaneously injected DC do not induce tumor immunity by antigen-independent mechanisms in this model. It is also unlikely that protection was the result of carryover of free SIINFEKL, as the peptide-pulsed DC were extensively washed before injection, and peptide with $\beta 2\text{M}$ alone is not protective when injected subcutaneously without DC (Fig. 2, B and D). Furthermore, mice immunized with SIINFEKL-pulsed DC were not protected from challenge with the untransfected parent B16 (Fig. 3, B and E), indicating that protective immunity was antigen specific, depending on OVA expression by the tumor target. Finally, we evaluated the contribution of CD8⁺ T cells to this protective tumor immunity by depleting groups of immunized or control animals of CD8⁺ effector cells before tumor challenge by repeated i.p. injection of anti-CD8 mAb (25, 30). Tumor growth and survival in immunized CD8⁺ T

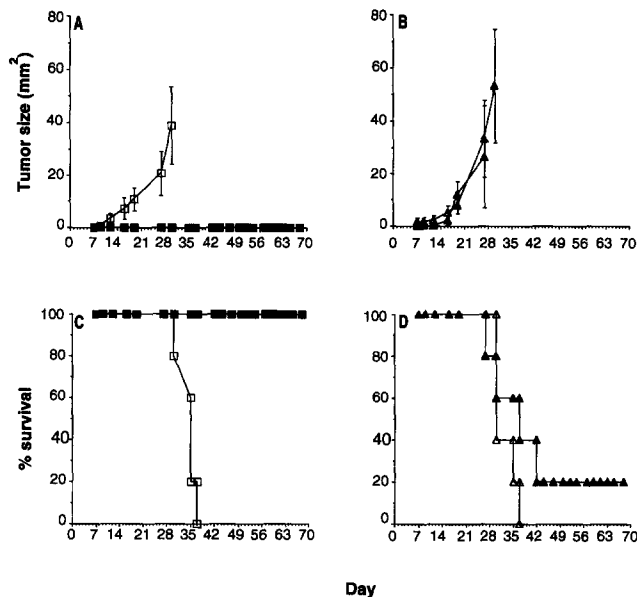


Figure 2. Immunization with peptide-pulsed DC induces protective immunity to lethal tumor challenge. C57BL/6 mice were immunized twice with PBS (open squares), peptide-pulsed DC (solid squares), peptide + β 2M (open triangles), or DC alone (solid triangles) on days 0 and 7. Mice were challenged with MO5 7 d after the last immunization (5×10^4 cells/mouse, i.d., bilateral, midflanks) (day 0). Tumor size (A and B) was assessed three times per week and is reported as the average tumor area in square millimeters until the first death occurred in each group. Survival (C and D) is recorded as the percentage of surviving animals. All experiments included five mice per group. Mice becoming moribund were killed.

cell-depleted animals was similar to that observed in non-immunized controls, with or without T cell depletion (Fig. 3, A, C, D and F). Therefore, CD8⁺ T cells are essential for the protective tumor immunity induced by peptide-pulsed DC in this model.

Interestingly, immunized mice that had rejected MO5

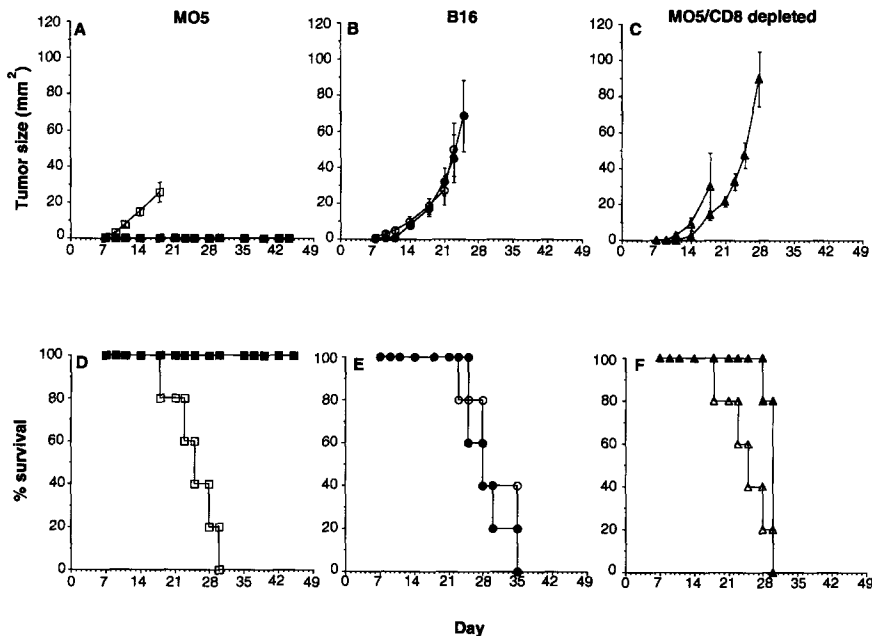


Figure 3. Tumor immunity induced by peptide-pulsed DC is antigen specific and CTL mediated. C57BL/6 mice were immunized twice with PBS (open symbols) or peptide-pulsed DC (solid symbols) on days 0 and 7. Some mice were depleted of CD8⁺ T lymphocytes by i.p. injection of anti-CD8 mAb 7 and 9 d after the last immunization (C and F). Mice were challenged with MO5 (A, C, D, and F) or B16 (B and E) as described (Fig. 2) 10 d after the last immunization (day 0). Tumor size (A–C) and survival (D–F) were recorded as described (Fig. 2). All experiments included five mice per group and were repeated at least three times. Mice becoming moribund were killed.

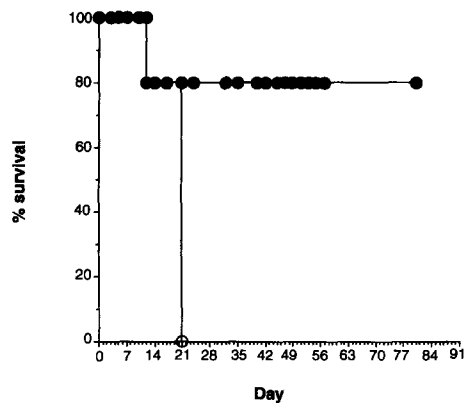


Figure 4. Immunization with peptide-pulsed DC and challenge with MO5 induces long-lasting protective immunity to B16. Naive mice (open circles) and surviving mice that had been immunized with peptide-pulsed DC (46 d previously) and challenged (solid circles) as described (Fig. 2) were rechallenged with the parental B16 melanoma (5×10^4 cells/mouse, i.d., bilateral, midflanks) (day 0). Survival is recorded as described (Fig. 2). Each group contained five mice. Experiments were repeated three times, and a representative experiment is shown. Mice that appeared moribund were killed.

were protected from subsequent tumor challenge by the untransfected parent B16 (Fig. 4). Presumably, mice rejecting the OVA-transfected melanoma developed immunity to other antigens expressed on MO5 and “shared” with the untransfected parent melanoma. Induction of immunity to shared tumor antigens has also been observed in this tumor model during tumor rejection after particulate antigen immunization (25). Immune responses to additional, undefined shared tumor antigens should augment antitumor immunity induced using defined antigens. Similarly, it may also be possible to induce immunotherapeutic responses by immunizing tumor-bearing hosts with DC pulsed with a potent peptide antigen and subsequently immunizing with their own killed tumor cells that have been transfected with

the antigen gene. This approach may be particularly significant for patients with tumors whose tumor rejection antigens have not yet been defined.

Several observations suggest that DC play a role in tumor immunity (31). Histologic infiltration of DCs into human tumors has been correlated with reduced metastatic disease and prolonged survival (32–36). In murine models, DC pulsed with tumor cell lysates can stimulate CD4-mediated antitumor immunity in vivo (37–40). To our knowledge, our studies are the first demonstration of CD8-mediated antitumor immunity induced by antigen-pulsed DC. Immunization with peptide alone or with adjuvants can induce antitumor immunity in murine models and is being evaluated in humans (41). Using DC as an adjuvant for peptide delivery has potential advantages over other forms of peptide delivery in that peptide in preformed complexes with class I on the surface of the DC is likely to be presented in the appropriate APC context for T cell stimulation and will be protected from degradation by extracellular proteases (42). It is possible that DC pulsing with

a combination of tumor cell lysates, which are processed and presented through the MHC class II-restricted pathway, and peptide antigens capable of forming complexes with cell surface class I molecules could enhance immunity by providing synergistic CD4- and CD8-mediated tumor-specific immunity.

DC used in our study were derived from bone marrow cultured in the presence of GM-CSF and IL-4 and express high levels of costimulatory molecules (15). When pulsed with peptides, these DC are more potent immunogens than bone marrow DC cultured in GM-CSF and TNF- α or GM-CSF alone (43). Interestingly, human DC can be readily obtained from peripheral blood by short-term in vitro culture with GM-CSF and IL-4 (13, 14). These human DC are phenotypically similar to those used in our study and can elicit potent antitumor CTL in vitro when pulsed with the relevant peptide (44). This may be significant for translational studies designed to induce immunity to human tumors.

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