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

By Christina M. Celluzzi,* Jose I. Mayordomo,* Walter J. Storkus,† Michael T. Lotze,‡ and Louis D. Falo, Jr.*

From the Departments of *Dermatology and †Surgery, Molecular Genetics, and Biochemistry, and the
Pittsburgh Cancer Institute, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania 15213

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.

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) (Kb 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 x 10⁶) 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 10⁶ cells/well. Cells were replated on day 1 at 2.5 x 10⁶ cells/well with GM-CSF (10³ U/ml; Sigma Chemical Co., St. Louis, MO) and murine rIL-4 (10³ U/ml; Genzyme Corp., Cambridge, MA), and loosely adherent cells were harvested on day 8. By flow cytometric analysis, these DC expressed CD45, CD44, CD1lb (Mac-1), CD18, CD80, CD86, and class I and class II MHC antigens (data not shown). DC were pulsed with 2 h at 37°C with or without OVA peptide (20 ng/ml) + ß₂-microglobulin ([ß₂M] 10 µg/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
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 \(\beta 2\)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 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 carriage or free SIINFEKL, as the peptide-pulsed DC were extensively washed before injection, and peptide with \(\beta 2\)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 + β2M (open triangles), or DC alone (solid triangles) on days 0 and 7. Mice were challenged with MO5 7 d after the last immunization (5 × 10⁴ 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.

Interestingly, immunized mice that had rejected MO5 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.

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 × 10⁴ 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 mouse 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.

This work was supported by grant AR011884 from the National Institutes of Health (L. D. Falo), a grant from the Dermatology Foundation (C. M. Celluzzi), and by an American Society of Clinical Oncology Young Investigators Award (J. I. Mayordomo).

Address correspondence to Dr. Louis D. Falo, Department of Dermatology, University of Pittsburgh School of Medicine, 190 Lothrop St., Pittsburgh, PA 15213.

Received for publication 21 July 1995 and in revised form 16 August 1995.

References

1. Doherty, P.C., B.B. Knowles, and P.J. Wettstein. 1984. Immunological surveillance of tumors in the context of major histocompatibility restriction of T-cell function. Adv. Cancer Res. 42:1–65.

2. Rosenberg, S.A., B.S. Packard, P.M. Aebersold, D. Solomon, S.L. Topalian, S.T. Toy, P. Simon, M. Lotze, J.C. Yang, C.A. Sepp, et al. 1988. Use of tumor infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. N. Engl. J. Med. 319:1676–1680.

3. Aebersold, P., C. Hyatt, S. Johnson, K. Hines, L. Korcak, M. Sanders, M. Lotze, S. Topalian, J. Yang, and S.A. Rosenberg. 1991. Lysis of autologous melanoma cells by tumor infiltrating lymphocytes: association with clinical response. J. Natl. Cancer Inst. 83:932–937.

4. Yewdell, J.W., and J.R. Bennink. 1992. Cell biology of antigen processing and presentation to major histocompatibility complex I molecule–restricted T-lymphocytes. Adv. Immunol. 52:1–123.

5. Townsend, A., and J. Trowsdale. 1993. The transporters associated with antigen presentation. Semin. Cell Biol. 4:53–61.

6. Slingluff, C.L., Jr., D.F. Hunt, and V.H. Englehard. 1994. Direct analysis of tumor-associated peptide antigens. Curr. Opin. Immunol. 6:733–740.

7. Pardoll, D.M. 1994. Tumour antigens. A new look for the 1990s. Nature (Lond.) 369:357–366.
murine bone marrow supplemented with GM-CSF and TNF-alpha. J. Immunother. 16:247 (Abstr.).

Inaba, K., R.M. Steinman, M.W. Pack, H. Aya, M. Inaba, T. Sudo, S. Wolpe, and G. Schuler. 1992. Identification of proliferating dendritic cell precursors in mouse blood. J. Exp. Med. 175:1157–1167.

Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R.M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J. Exp. Med. 176:1693–1702.

Bastin, J., J. Rothbard, J. Davey, J. Jones, and A. Townsend. 1987. Use of synthetic peptides of influenza nucleoprotein to define epitopes recognized by class I-restricted cytotoxic T lymphocytes. J. Exp. Med. 165:1508–1523.

Inaba, K., J.W. Young, and R.M. Steinman. 1987. Direct activation of CD8+ cytotoxic T lymphocytes by dendritic cells. J. Exp. Med. 166:182–194.

Macatonia, S.E., P.M. Taylor, S.C. Knight, and B.A. Askonas. 1989. Primary stimulation by dendritic cells induces antiviral proliferative and cytotoxic T cell responses in vitro. J. Exp. Med. 169:1255–1264.

Inaba, K., J.P. Metlay, M.T. Crowley, and R.M. Steinman. 1990. Dendritic cells pulsed with protein antigens in vitro can prime antigen-specific, MHC-restricted T cells in situ. J. Exp. Med. 172:631–640.

Townsend, A., T. Elliot, V. Cerundolo, L. Foster, B. Barber, and A. Tse. 1990. Assembly of MHC class I molecules analyzed in vitro. Cell. 62:285–295.

Falk, K., O. Lützschke, S. Stevanovic, G. Jung, and H.G. Schuler. 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature (Lond.). 351:290–296.

Rock, K.L., L. Lammensee, and S. Gamble. 1993. Serum proteases alter the antigenicity of peptides pulsed antigen-presenting cells. J. Immunol. 142:1053–1059.

Rock, K.L., D.D. Jr., L.J. Colarusso, B. Benacerraf, and K.L. Rock. 1995. Targeting antigen into the phagocytic pathway in vivo induces protective tumour immunity. Nature Med. 1:649–653.

Falk, K., O. Rötzschke, S. Stevanovic, G. Jung, and H.G. Schuler. 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature (Lond.). 351:290–296.

Rock, K.L., L. Rothstein, and S. Gamble. 1990. Generation of class I MHC-restricted T-1 hybridomas. J. Immunol. 145:804–811.

Rock, K.L., C. Fleischacker, and S. Gamble. 1993. Peptide priming of cytolytic T cell immunity in vivo using β2-microglobulin as an adjuvant. J. Immunol. 150:1244–1251.

Benjamin, R.J., J.A. Madrigal, and P. Parham. 1991. Peptide binding to empty HLA-B27 molecules of viable human cells. Nature (Lond.). 351:74–77.

Auchincloss, H.J., R.R. Ghobrial, P.S. Russell, and H.J. Winn. 1988. Prevention of alloantibody formation after skin grafting without prolongation of graft survival by anti-L3T4 in vivo. Transplantation. 45:1118–1123.

Grabbe, S., S. Beisert, T. Schwarz, and R.D. Granstein. 1995. Dendritic cells as initiators of tumor immune responses: a possible strategy for tumor immunotherapy? Immunol. Today. 16:117–120.

Tazi, A., F. Bouchonnet, M. Grandsaigne, L. Boumell, A.J. Hance, and P. Soler. 1993. Evidence that granulocyte macrophage-colony-stimulating factor regulates the distribution and differentiated state of dendritic cells/Langerhans cells in human lung and lung cancers. J. Clin. Invest. 91:566–576.

Becker, Y. 1993. Dendritic cell activity against primary tumors: an overview. In Vivo. 7:187–191.

Azizi, E., C. Bucana, L. Goldberg, and M.L. Kripke. 1987. Perturbation of epidermal Langerhans cells in basal cell carcinomas. Am. J. Dermatopathol. 9:465–473.

Murphy, G.F., A. Radu, M. Kaminer, and D. Berd. 1993. Autologous melanoma vaccine induces inflammatory responses in melanoma metastases: relevance to immunologic regression and immunotherapy. J. Invest. Dermatol. 100:335–341S.

Tsujitani, S., Y. Kakceji, A. Watanabe, S. Kohnoe, Y. Maebara, and K. Sugimachi. 1990. Infiltration of dendritic cells in relation to tumor invasion and lymph node metastasis in human gastric cancer. Cancer. 66:2012–2016.

Grabbe, S., S. Bruvers, R.L. Gallo, T.L. Knisely, R. Nazereno, and R.D. Granstein. 1991. Tumor antigen presentation by murine epidermal cells. J. Immunol. 146:3656–3661.

Cohen, P.J., P.A. Cohen, S.A. Rosenberg, S.I. Katz, and J.J. Mule. 1994. Murine epidermal Langerhans cells and splenic dendritic cells present tumor-associated antigens to primed T cells. Eur. J. Immunol. 24:315–319.

Plmand, V., T. Sornasse, K. Thieleman, D. Demonet, M. Bakkus, H. Bazin, F. Tieleman, O. Leo, J. Urbain, and M. Moser. 1994. Murine dendritic cells pulsed in vitro with tumor antigen induce tumor resistance in vivo. Eur. J. Immunol. 24:605–610.

Zou, J.P., J. Shimizu, K. Ikegame, N. Yamamoto, S. Ono, H. Fujiwara, and T. Hamaoka. 1992. Tumor-bearing mice exhibit a progressive increase in tumor antigen-presenting cell function and a reciprocal decrease in tumor antigen-responsive CD4+ T cell activity. J. Immunol. 148:648–655.

Shimizu, J., T. Suda, T. Yoshikata, A. Kosugi, H. Fujiwara, and T. Hamaoka. 1989. Induction of tumor-specific in vivo protective immunity by immunization with tumor antigen-pulsed antigen-presenting cells. J. Immunol. 142:1053–1059.

Falo, L.D., Jr., L.J. Colarusso, B. Benacerraf, and K.L. Rock. 1992. Serum proteases alter the antigenicity of peptides presented by class I major histocompatibility complex molecules. Proc. Natl. Acad. Sci. USA. 89:8347–8350.

Mayordomo, J.I., T. Zorina, W.J. Storkus, L. Zitvogel, C. Celluzzi, L.D. Falo, C.J. Melief, S.T. Ildstad, W. Martin Kast, A.B. DeLeo, and M.T. Lotze. 1995. Bone marrow–derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. Nature Med. 1:1297–1302.

Mayordomo, J.I., L. Zitvogel, T. Tjandrawan, M.T. Lotze, and W.J. Storkus. 1995. Dendritic cells presenting tumor peptide epitopes stimulate effective anti-tumor CTL in vitro and in vivo. Proc. Int'l Assoc. Immunol. In press.