Epidermal Dendritic Cells Induce Potent Antigen-Specific CTL-Mediated Immunity

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Professional antigen-presenting cells (APCs) are required for the initiation of an immune response. Dendritic cells (DCs) are the most potent APCs identified thus far and can present antigen in the context of co-stimulatory signals required for the stimulation of both primed and naïve T cells. Cytotoxic T lymphocytes (CTLs) are critical to the immune response against tumors or virally infected cells. Optimal stimulation of antigen-specific CTLs is the goal of evolving immunization strategies for the prevention or therapy of viral infections and tumors. Epidermal dendritic cells (eDCs), or Langerhans cells, can present antigens for the stimulation of $CD4^+$ T cell dependent anti-tumor immunity and may play a role in tumor surveillance. The capacity of eDCs to induce tumor-specific $CD8^+$ CTL immunity has not been determined. We have previously shown that DCs derived from bone marrow precursors (BmDCs) under the influence of cytokines can induce protective, antigen-specific CTL-mediated anti-tumor immunity. Here we show that subcutaneous immunization with ovalbumin (OVA) peptide ($SIINFEKL_{257-264}$)-pulsed eDCs induced OVA-specific, $CD8^+$ CTLs that lyse the OVA-expressing target. Furthermore, mice vaccinated with OVA peptide–pulsed eDCs were completely protected from subsequent challenge by the OVA-expressing melanoma MO5. The capacity of peptide-pulsed eDCs to induce CTL-mediated immunity is directly dependent on the dose of eDCs administered. Importantly, the APC capacity of eDCs is comparable to that of BmDCs, as mice immunized with eDC populations containing at least as many class $II^+/B7.2^+$ cells as populations of BmDCs were equally protected against challenge with MO5. These results demonstrate that eDCs can be potent inducers of antigen-specific $CD8^+$ CTL-mediated immunity. They suggest that eDCs may be important targets for antigen delivery strategies aimed at inducing antiviral or anti-tumor immunity. *Key words: $CD8^+$ T cells/vaccine/Langerhans cells. J Invest Dermatol 108:716–720, 1997*

Cytotoxic T lymphocytes (CTLs) play an important role in the elimination of tumors and virally infected cells (Fast and Fan, 1981, Doherty *et al*, 1984). The induction of CTLs depends on the recognition of cell surface peptide–major histocompatibility complex (MHC) class I complexes and the delivery of co-stimulatory signals by professional antigen-presenting cells (APCs). The delivery of antigen in a manner that will facilitate antigen-specific stimulation of T cells is a critical challenge in vaccine design.

One approach to vaccine design is to develop strategies to deliver antigen via professional APCs. Dendritic cells (DCs) are the most potent APCs identified to date. They are defined morphologically by their unique "veiled" appearance, functionally by their potent capacity to stimulate alloreactive T cells and their ability to prime naïve T cells *in vitro* and *in vivo* (Romani *et al*, 1989; see Steinman, 1991), and phenotypically by their high levels of expression of MHC class II and co-stimulatory molecules including CD80 and CD86. The skin is rich in DCs. Epidermal DCs (eDCs), or Langerhans cells, are capable of taking up antigen in the skin, and then migrating to the regional lymph nodes where they can efficiently stimulate T lymphocytes (Romani and Schuler, 1992). Through this "sentinel" function, eDCs perform a critical immunoregulatory function; they deliver foreign antigens from the periphery to the central lymphoid organs in the appropriate APC context for the induction of effector T lymphocytes. This function, in combination with their unique accessibility, suggests that eDCs may be important targets for antigen delivery strategies aimed at inducing immunity against infectious diseases and tumors.

In vitro and *in vivo* studies have demonstrated that Langerhans cells can present alloantigens, protein antigens, and haptens to lymphocytes (Shimizu *et al*, 1989; Romani and Schuler, 1992). Murine models suggest that eDCs are capable of inducing $CD4^+$ T cell-dependent immunity to tumor-associated antigens and may play a role in anti-tumor surveillance (Grabbe *et al*, 1991; Cohen *et al*, 1994). The capacity of eDCs to induce tumor-specific $CD8^+$ CTL immunity has not been determined.

Our studies and others have demonstrated that BmDCs preloaded with antigen and injected to a host are able to induce antigen-specific $CD8^+$ CTLs, which can mediate both protective and therapeutic immunity to tumors (Mayordomo *et al*; 1995,

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Abbreviations: CTLs, cytotoxic T lymphocytes; APCs, antigen-presenting cells; DCs, dendritic cells; eDCs, epidermal-derived dendritic cells; BmDCs, bone marrow–derived dendritic cells; OVA, chicken egg albumin; β2M, β2-microglobulin.

Ossevoort *et al*, 1995; Porgador and Gilboa, 1995; Celluzzi *et al*, 1996; Porgador *et al*, 1996). eDCs, like DCs derived from bone marrow precursors in the presence of cytokines, develop dendritic morphology and exhibit enhanced surface expression of MHC class I and II antigen and important co-stimulatory molecules after 2–3 d in culture (Cohen *et al*, 1994; Razi-Wolf *et al*, 1994). This "maturation" occurs during culture in the presence of exogenously added cytokines or in the presence of cytokines provided by co-cultured keratinocytes (Luger, 1989). Recent studies suggest that different pathways of DC development exist (Caux *et al*, 1996; Peters *et al*, 1996). Interestingly, although DCs derived from skin and "matured" by co-culture with keratinocytes appear to be phenotypically similar to "mature" DCs derived from bone marrow in the presence of exogenously added cytokines, functional comparisons of epithelial *versus* bone marrow-derived DCs have not yet been performed. In this study, we investigate the capacity of eDCs to induce antigen-specific $CD8^+$ CTL-mediated anti-tumor immunity and directly compare the antigen presentation capacity of mature DCs derived from epidermal and bone marrow precursors.

MATERIALS AND METHODS

Mice and Cell Lines Female C57BL/6 mice 5–8 wk of age were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed at the Central Animal Facility of the University of Pittsburgh. RF33.70 is a C57BL/6-derived anti-OVA + K^b specific hybridoma described previously (Rock *et al*, 1990b). EL4 is a C57BL/6 T lymphoma ($H\text{-}2^b$), and EG7 is a chicken egg albumin (OVA)-transfected subclone of EL4 (Moore *et al*, 1988). The MO5 was constructed by transfection of the C57BL/6-derived murine melanoma, B16 ($H\text{-}2^b$) (ATCC, Rockville, MD), with the pAc-neo-OVA plasmid, as described (Falo *et al*, 1995), which encodes a dominant $H\text{-}2^b$–restricted CTL epitope (SIINFEKL). SIINFEKL serves as the tumor rejection antigen to which an immune response is elicited (Falo *et al*, 1995).

Antigen and Antibodies The peptide corresponding to the amino acid sequence of OVA residues 257–264 (SIINFEKL) (K^b restricted) (Rock *et al*, 1990a; Falk *et al*, 1991) was synthesized by the Peptide Synthesis Facility of the University of Pittsburgh Medical Center. Monoclonal antibodies used to deplete cell subsets were prepared from the hybridomas GK1.5 (anti-CD4, ATCC TIB 207), 2.43 (anti-CD8, ATCC TIB 210), 30-H12 (anti-Thy 1.2, ATCC TIB 107), B220 (anti-B cell surface glycoprotein, ATCC TIB 146), and NK1.1 (kindly provided by W. Chambers, University of Pittsburgh School of Medicine). Fluoroscein isothiocyanate-conjugated mouse anti-mouse $I\text{-}A^{b,d}$ and phycoerythrin-conjugated rat anti-mouse B7.2 monoclonal antibodies (Pharmingen, San Diego, CA) were used to phenotype dendritic cells.

Preparation of Epidermal-Derived DCs (eDCs) DCs were obtained as described (Razi-Wolf *et al*, 1994). Briefly, ears from C57BL/6 mice were separated and floated onto dispase (1.6 U per ml, Boehringer Mannheim, Indianapolis, IN) at 37°C. After a 1-h incubation, epidermal sheets were removed and resuspended in trypsin (0.05%)/DNase (160 μg per ml) for 15 min at 37°C. Cell suspensions were obtained by dissociating tissue by scraping with forceps and passing it through a nylon filter (Falcon) to remove hair and debris. Cells were resuspended in RPMI 1640 (10% fetal bovine serum, L-glutamine, antibiotics, 2mercaptoethanol), and incubated at 37°C (10% CO_2) for 2–3 d (the time at which cells reach maturity). Granulocyte-macrophage colony-stimulating factor (GM-CSF) was supplied by keratinocytes present in the culture (Luger, 1989). Nonadherent cells were enriched over a density gradient (Lymphoprep, Nycomed, Norway) and washed extensively before use. As determined by flow cytometric analysis, approximately 35% of eDCs expressed class II MHC antigens ($I\text{-}A^+$). Approximately 6–10% of the eDCs expressed both CD86 (B7.2) and class II MHC antigens ($I\text{-}A^+$) (data not shown).

Preparation of Bone Marrow–Derived DCs (BmDCs) Dendritic cells were prepared from bone marrow as described (Celluzzi *et al*, 1996). Briefly, bone marrow cells were depleted of lymphocytes and cultured overnight at 10^6 cells per ml in RPMI 1640. Cells were replated on day 1 at 2.5×10^5 cells per ml with GM-CSF (10^3 U per ml; Sigma Chemical Co., St. Louis, MO) and murine rIL4 (500 U per ml; Sigma). Loosely adherent cells were harvested on day 8. By flow cytometric analysis, 50–75% BmDCs co-expressed B7.2 and class II MHC ($I\text{-}A^+$) antigens (data not shown).

T-T Hybridoma Assay Microcultures (200 μl/well, 96-well flat bottom plates) were prepared with eDCs or BmDCs (10^5 cells/well) and RF33.70 (10^5 cells/well) in the presence or absence of the indicated concentration of OVA peptide (**Fig 1**). After 18 h incubation at 37°C, 100 μl of the supernatants were collected and assayed for lymphokine using the interleukin (IL)-2–dependent indicator cell line HT2 (Watson, 1979). The results are expressed as mean counts per minute of duplicate cultures. Assays were repeated at least three times.

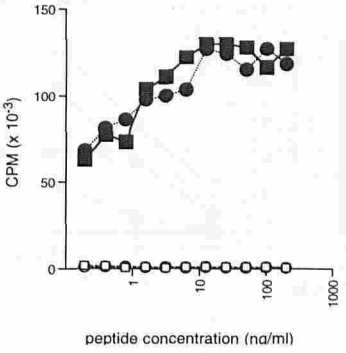

Figure 1. Presentation of exogenous peptide by epidermal (eDCs) and bone marrow-derived dendritic cells (BmDCs). eDCs *(squares)* or BmDCs *(circles)* were co-cultured with the T-cell hybridoma RF33.70 (anti-OVA $+K^b$) (Rock *et al*, 1990b) in the absence *(open symbols)* or presence *(filled symbols)* of OVA peptide (titrated as indicated). After 18 h, supernatants were harvested and assayed for lymphokine. Values are given as counts per min of duplicate cultures.

Cytotoxicity Assay Splenocytes (30×10^6), harvested from mice 7–10 d after the last immunization, were restimulated by co-culture with irradiated EG7 cells (20,000 rad, 7.5×10^6) for 5 d. After this time, cytotoxicity assays were performed as described (Celluzzi *et al*, 1996). Eighteen hours prior to assay, target cells were labeled by incubation in RPMI with ^{51}Cr (100 μCi; NEN, Boston, MA) at 37°C and washed extensively before use. Nondepleted or monoclonal antibody + C′–treated splenocyte effectors depleted for $CD4^+$, $CD8^+$, Thy 1.2^+, or NK^+ cells were co-cultured at 37°C in 96-well round bottom plates for 4 h at the indicated ratios in RPMI with either ^{51}Cr-labeled OVA-expressing EG7, or non–OVA-expressing EL4 target cells (2×10^4 targets/well). One hundred microliters of supernatants from triplicate cultures were collected and counted. Data points are expressed as the mean percent specific release of ^{51}Cr from target cells and were calculated as described (Rock *et al*, 1993b).

T-Cell Activation Assay Microcultures (5×10^5-10^6 cells per well, 200 μl/well) were prepared with splenocytes from immunized or control mice plus irradiated OVA-expressing EG7 cells in RPMI containing 1% normal mouse serum or medium alone in 96 well round-bottom plates and cultured for 48 h at 37°C. Supernatants (100 μl) were harvested and assayed for lymphokine activity on HT2 cells as described above. The results are expressed as mean counts per min of triplicate cultures. Irradiated EG7 cells alone were included to determine background lymphokine stimulation levels (data not shown).

Immunization and Protection Assays eDCs or BmDCs were pulsed for 2 h at 37°C with SIINFEKL OVA peptide (20 ng per ml) + β2-microglobulin (β2M) (10 μg per ml, human, Sigma) (Rock *et al*, 1993a) in reduced serum medium (Optimem; GIBCO Laboratories, Grand Island, NY). Cells were then washed extensively and resuspended in phosphate-buffered saline (PBS). C57BL/6 mice were immunized subcutaneously in both lower flanks with peptide-pulsed eDCs at two concentrations (1×10^6 or 6×10^4 per mouse/200 μl at 100 μl/side), peptide-pulsed BmDCs (6×10^4/mouse/200 μl at 100 μl/side), peptide (20 ng per ml) or PBS on day 0. Seven days later, mice were challenged by intradermal injection in the midflanks bilaterally with MO5 (5×10^4/mouse/200 μl at 100 μl/side) in PBS. The size of the tumors was assessed two to three times weekly and recorded as tumor area (mm^2) by measuring the largest perpendicular diameters. Data are reported as the average tumor area ± SEM and are plotted until the first mouse in each test group is sacrificed. Survival is recorded as the percentage of surviving animals. All experiments included five mice per group and were repeated. Mice becoming moribund were sacrificed. All peptide pulsing was done in the presence of exogenous human β2M.

RESULTS

Exogenous Peptide Accesses Class I on the Surface of Cultured eDCs CTLs have been found to play an important role in the elimination of tumors. To generate CTL-mediated immunity, antigens must be presented by MHC class I molecules. Access to this pathway is typically limited to endogenous antigen. Recent studies have shown, however, that exogenously added peptide

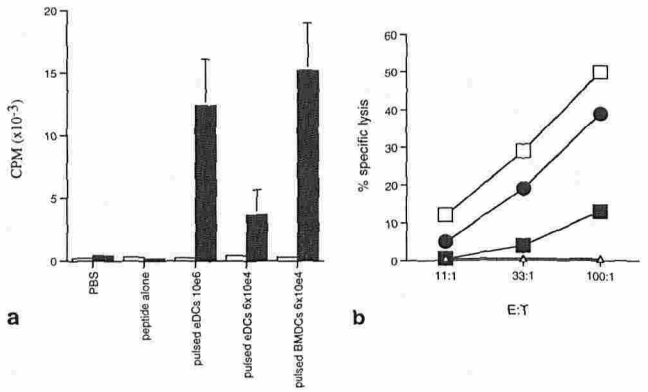

Figure 2. Mice immunized with OVA peptide-pulsed epidermal-derived dendritic cells (eDCs) prime specific T-cell responses. *Splenocytes from immunized mice were harvested and restimulated in flasks or microtiter plates in the presence of irradiated EG7. (a)* Supernatants from splenocytes stimulated 48 h in microtiter plates were assayed for lymphokine by culture with HT2 cells. Immunization conditions of the mice are shown along the *x* axis. Proliferation of HT2 cells in the presence of supernatant is shown for splenocytes cultured with *(filled bars)* or without *(open bars)* irradiated EG7 stimulators. Supernatants from culture of EG7 alone did not support HT2 proliferation (not shown). *(b)* Splenocytes from mice immunized with PBS (△), OVA peptide-pulsed eDCs [6×10^4 cells/mouse (■), 1×10^6 cells/mouse (□)], or OVA peptide-pulsed BmDCs [6×10^4 cells/mouse (●)] were assayed for their ability to lyse ^{51}Cr-labeled OVA-expressing at the effector to target ratios shown. Results are reported as percent specific ^{51}Cr release and are the mean of triplicate cultures. Less than 10% lysis was observed in non-OVA–expressing targets at all ratios (not shown).

antigen can gain access to this pathway by binding to class I molecules on the surface of APCs (Rock *et al*, 1990a, Celluzzi *et al*, 1996). To assess the ability of eDCs to present functional peptide + K^b complexes on the cell surface, eDCs or BmDCs were cultured *with or without the OVA peptide, SIINFEKL, in the presence of* the OVA + K^b-specific cell hybridoma RF33.70. Cells that present the OVA peptide in association with its surface class I molecules stimulate RF33.70 to produce IL-2, which is measured by proliferation of the IL-2–dependent indicator cell line, HT2. eDCs were able to present functional SIINFEKL + K^b complexes on the cell surface in the presence of exogenous peptide, at concentrations as low as 0.19 ng per ml, to RF33.70 **(Fig 1)**. Furthermore, presentation of exogenous peptide by eDCs was similar to that observed for BmDCs. eDCs and BmDCs cultured in the absence of peptide did not stimulate IL-2 production by RF33.70 **(Fig 1)**.

Immunization with OVA Peptide-Pulsed eDCs Primes OVA-Specific T-Cell Responses To evaluate T-cell priming in immunized mice, we measured T-cell activity by lymphokine production and lytic activity. To determine whether T-cell responses depended on the number of MHC class $II^+/B7.2^+$ cells used to immunize, two doses of eDCS ($1 \times 10^6/6 \times 10^4$) were compared to BmDCs (6×10^4). Flow cytometric studies showed that approximately 6–10% of eDCs co-expressed B7.2 and class II molecules, whereas 50–75% of BmDCs were MHC class $II^+/B7.2^+$ (data not shown). The amount 6×10^4 was selected for comparison because it corresponded to the absolute number of BmDCs that in earlier experiments could induce antigen-specific CTLs (Celluzzi *et al*, 1996), whereas 1×10^6 was selected to incorporate at least as many class $II^+/B7.2^+$ eDCs as BmDCs.

To measure SIINFEKL + K^b–dependent secondary stimulation, splenocytes from mice immunized with either 6×10^4 or 1×10^6 peptide-pulsed eDCs, peptide-pulsed BmDCs (6×10^4), peptide alone, or PBS were cultured in the presence or absence of irradiated OVA-expressing EG7 cells, and supernatants were assayed with HT2 cells to determine the presence of lymphokine **(Fig 2*a*)**. Splenocytes from mice immunized with peptide-pulsed eDCs at 1×10^6, or BmDCs at 6×10^4, demonstrated similar levels of OVA-specific activation. Neither PBS-injected control mice nor mice immunized with peptide alone had measurable antigen-specific T cell activity in this assay. Immunization with 6×10^4 peptide-pulsed eDCs results in measurable OVA-specific activity that was significantly less than that observed in animals immunized with 1×10^6 peptide-pulsed eDCs.

Table I. Immunization with OVA Peptide-pulsed eDCs induces $CD8^+$ CTLs

Effector: Target	% Specific Lysis of OVA-Expressing Target Cells from Splenocytes				
	Nondepleted	CD4-	CD8-	NK-	Thy1-
1:1	0[a] (0)[b]	0 (0)	0 (0)	0 (0)	0 (0)
25:1	28 (10)	30 (13)	0 (0)	29 (13)	0 (0)
50:1	74 (28)	—[c]	—	—	—

[a] Lysis observed from splenocytes harvested from mice immunized with 1×10^6 OVA peptide-pulsed eDCs. Mean ± 2%.
[b] Lysis observed from splenocytes harvested from mice immunized with 6×10^4 OVA peptide-pulsed eDCs. Mean ± 2%.
[c] Not tested.

OVA peptide-pulsed BmDCs induce OVA-specific $CD8^+$ CTLs (Celluzzi *et al*, 1996). Effector cells obtained from mice immunized with 6×10^4 peptide-pulsed BmDCs were able to lyse OVA-expressing tumors (EG7, MO5), but not the untransfected parents (EL4, B16) (Celluzzi *et al*, 1996). To evaluate and compare the ability of eDCs to induce OVA-specific CTLs, mice were immunized, as above, with varying numbers of eDCs pulsed with OVA peptide (either 6×10^4 or 1×10^6 eDCs), peptide-pulsed BmDCs at 6×10^4 cells, peptide alone, or PBS.

In vitro restimulated spleen cells from mice immunized with 1×10^6 peptide-pulsed eDCs lysed the OVA-transfected EG7 cells at levels comparable to those from mice immunized with 6×10^4 peptide-pulsed BmDCs. Furthermore, lysis was $CD8^+$ T cell mediated **(Table I)**. Splenocytes depleted of CD8+ T cells were unable to lyse EG7 targets whereas splenocytes depleted of CD4 or NK cells retained the ability to lyse EG7 targets compared to nondepleted controls. Splenocytes from animals immunized with fewer peptide-pulsed eDCs (6×10^4) or PBS did not **(Fig 2*b*)** lyse EG7 targets at comparable levels. Spleen cells from immunized animals did not lyse the untransfected parent tumor, EL4 (data not shown), demonstrating that lysis of the tumor is antigen specific, depending on expression of OVA by the tumor target.

Immunization with Peptide-Pulsed eDCs Cells Induces Protective Immunity to the OVA-Transfected Melanoma, MO5 Immunization with peptide-pulsed BmDCs (6×10^4 cells) fully protect mice from challenge with the OVA-transfected melanoma, MO5, but not from the parent B16 (Celluzzi *et al*, 1996). Moreover, neither BmDCs alone nor peptide alone were able to protect mice from tumor challenge, indicating that peptide-pulsed BmDCs (6×10^4) were necessary to prime the antigen-specific protective response (Celluzzi *et al*, 1996). To compare the capacity of eDCs to BmDCs to induce protective immunity, groups of mice were immunized, as described above, with either peptide-pulsed 1×10^6 or 6×10^4 eDCs, peptide-pulsed BmDCs, peptide alone, or PBS. As expected, 100% of mice immunized with peptide-pulsed BmDCs were protected from tumor challenge **(Fig 3*a,b*)**. Interestingly, mice immunized with peptide-pulsed 1×10^6 eDCs were also completely protected, whereas 60% of mice receiving 6×10^4 peptide-pulsed eDCs developed lethal tumors. By contrast, all mice receiving PBS or peptide alone developed lethal tumors. These results indicate that peptide-pulsed eDCs induce protective tumor immunity. Furthermore, they demonstrate that the effectiveness of immunization depends on the number of eDCs administered.

DISCUSSION

The processing and presentation of antigen that is required for immune responsiveness can occur through two pathways. Typi-

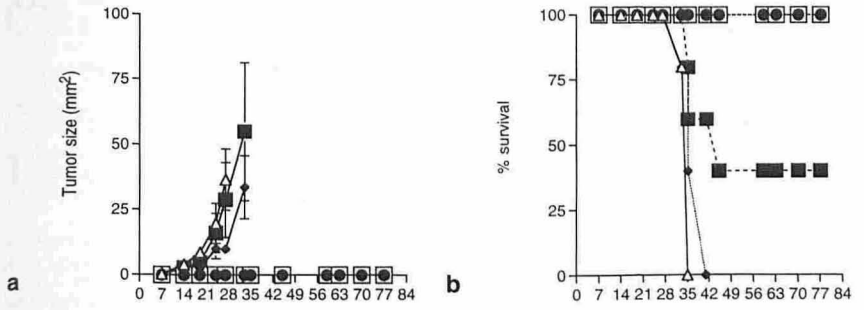

Figure 3. Immunization with a threshold level of peptide-pulsed epidermal-derived dendritic cells (eDCs) induces protective immunity to the OVA-transfected melanoma, MO5. C57BL/6J mice were immunized with PBS (△), OVA peptide (◆), OVA peptide-pulsed eDCs [6×10^4 cells/mouse (■) or 1×10^6 cells/mouse (□)], or OVA peptide-pulsed BmDCs (6×10^4 cells/mouse, ●). Seven days later, mice were challenged with MO5. The size *(a)* of each tumor is reported as the area ± SEM in square millimeters. Survival *(b)* is recorded as the percentage of surviving animals. Immunization with unpulsed dendritic cells alone is not protective (data not shown, Celluzzi *et al*, 1996). n = 5 for all experiments.

cally, in the MHC class I pathway, intracellular or endogenously synthesized antigens, such as viruses, are reduced to peptides by proteasomes in the cytosol. Peptides are carried by the transporter in antigen processing into the endoplasmic reticulum where they form a MHC class I-peptide–β2M complex that is routed through the Golgi complex to the surface of the cell (Morrison *et al*, 1986; Moore *et al*, 1988). Antigen synthesized within the cell is recognized by MHC class I–restricted $CD8^+$ CTLs resulting in lysis of the target cell. In contrast, extracellular or exogenous proteins are generally processed via the MHC class II pathway. Peptides derived from extracellular proteins in phagolysosomes outside of the cytosol bind to MHC class II molecules to form MHC class II-peptide complexes that can be recognized by $CD4^+$ T helper cells.

In the elimination of tumors, induction of CTLs plays an important role. Optimal stimulation of an antigen-specific CTL response is the aim of strategies designed for the prevention and treatment of tumors. Although exogenous proteins are generally excluded from the class I presenting pathway, an approach to target antigen into the class I pathway is to target exogenously added peptides to accessible class I molecules on the surface of professional APCs. APCs contain class I molecules that are accessible to exogenously added peptide (Rock *et al*, 1993a, 1993b). In the presence of exogenously added β2M, functional complexes consisting of class I heavy chain, exogenous peptide, and β2M are present on the surfaces of APCs (Rock *et al*, 1993a, 1993b). Using this strategy, professional APCs, which can also provide necessary co-stimulatory signals for the expansion of CTLs, were loaded *ex vivo* with peptide before injection to a host.

BmDCs, tested in earlier studies, were able to present the exogenously added OVA peptide, SIINFEKL, with surface class I molecules (K^b). SIINFEKL-pulsed BmDCs injected subcutaneously induced antigen-specific, $CD8^+$ CTL-mediated protective immunity to MO5, an OVA-transfected, C57BL/6 mouse-derived, B16 melanoma. In the current studies, we used the same B16/MO5 model to compare the ability of eDCs and BmDCs to present exogenously added peptide to an MHC class I-restricted hybrid and to induce antigen-specific CTL-mediated immunity.

Maturation and development of cultured DCs is mediated by exogenously added GM-CSF or GM-CSF produced by keratinocytes present in culture (Heufler *et al*, 1988; Luger, 1989; Inaba *et al*, 1992). In our studies BmDCs were cultured in the presence of both GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994) whereas eDCs were grown in the presence of keratinocytes that provide a source of cytokines including GM-CSF (Luger, 1989). It is also important to consider that both eDC and BmDC preparations are heterogenous cell populations that contain cell types other than dendritic cells. Nonetheless, BmDCs and eDCs in our studies resembled each other morphologically, phenotypically, and functionally.

In vitro studies demonstrate that even when small amounts of exogenous SIINFEKL peptide was added (<1 ng per ml) to eDCs, functional OVA + K^b complexes formed on the cell surface, at levels similar to BmDCs **(Fig 1)**, indicating that these cells have comparable levels of MHC class I molecules that are accessible to exogenously added peptides.

Furthermore, OVA peptide-pulsed eDCs induce OVA-specific T-cell immunity **(Fig 2*a,b*)**. The effectiveness of this form of immunization depends on the number of peptide-pulsed eDCs used to immunize. Splenocytes harvested from mice immunized with eDCs adjusted to include at least as many APCs as in the BmDCs preparation (1×10^6 eDCs = 1×10^5 class $II^+/B7.2^+$ cells), show similar levels of OVA-specific activation and lytic activity. Limited target lysis is observed from mice immunized with fewer (6×10^4) peptide-pulsed eDCs ($6 \times 10^4 = 6 \times 10^3$ class $II^+/B7.2^+$ cells). Mice immunized with peptide alone did not produce significant responses in either assay **(Fig 2)**.

Both cytolytic activity and lymphokine responses correlated with *in vivo* protection. All mice (100%) immunized with 1×10^6 peptide-pulsed eDCs or 6×10^4 peptide-pulsed BmDCs were protected from tumor challenge **(Fig 3*a,b*)**. By contrast, 40% of mice immunized with fewer (6×10^4) peptide-pulsed eDCs were protected. The partial protection observed in this group, coupled with the intermediate lymphokine production and CTL activity observed, suggests that this number of eDCs may be on the threshold between an effective and ineffective immunizing dose. Our results, using defined antigens, support previous studies that demonstrate that epidermal Langerhans cells pulsed with tumor lysates stimulate protective tumor immunity (Grabbe *et al*, 1991; Cohen *et al*, 1994) and demonstrate the induction of antigen-specific CTLs.

Peptide loading onto DCs might offer several advantages over the administration of free peptide or antigen. Because the peptide is presented on a complex on the cell surface, the peptide avoids degradation by extracellular proteases (Falo *et al*, 1992). In addition, no free peptide is available to bind nonprofessional APCs. Free peptide administered alone could target healthy cells for destruction, or bind to nonprofessional APCs, which lacking co-stimulatory molecules, may result in downregulation rather than stimulation.

Immunogenicity of dendritic cells depends presumably on a number of factors including (i) the effectiveness of antigen loading, (ii) the delivery of co-stimulatory signals and (iii) trafficking of antigen-loaded cells on readministration. It is likely that several variables, including the origin of the dendritic cells, their culture conditions, which affect the expression/regulation of relevant co-stimulatory molecules, their routes of administration, and their dose, will affect immunogenicity.

We have previously shown that *in vitro* culture conditions used to generate BmDCs can effect their immunogenicity *in vivo* (Mayordomo *et al*, 1995). The studies reported here directly compare the immunogenicity of eDCs with BmDCs. They demonstrate that like BmDCs, peptide-pulsed eDCs can induce antigen-specific $CD8^+$ CTLs and tumor immunity if similar numbers of class $II^+/B7.2^+$ cells are present. Thus, eDCs appeared functionally similar to BmDCs in our experiments. We have recently reported that delivery of antigen-encoding genes to cutaneous DCs *in vivo* results in the induction of potent antigen-specific CTL-mediated immunity (Condon *et al*, 1996). Taken together, these studies provide rationale for the development of immunization strategies that target antigen delivery to cutaneous DCs *in vitro* or *in vivo*.

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