Bone Marrow–generated Dendritic Cells Pulsed with a Class I–restricted Peptide Are Potent Inducers of Cytotoxic T Lymphocytes

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Summary

It has previously been shown that bone marrow–generated dendritic cells (DC) are potent stimulators in allogeneic mixed leukocyte reactions and are capable of activating naive CD4+ T cells in situ in an antigen-specific manner. In this study we have investigated whether bone marrow–generated DC are capable of inducing antigen-specific CD8+ T cell responses in vivo. Initial attempts to induce specific cytotoxic T lymphocyte (CTL) responses in mice injected with bone marrow–generated DC pulsed with ovalbumin (OVA) peptide were frustrated by the presence of high levels of nonspecific lytic activity, which obscured, though not completely, the presence of Ag-specific CTL. Using conditions that effectively differentiate between antigen-specific and nonspecific lytic activity, we have shown that bone marrow–generated DC pulsed with OVA peptide are potent inducers of OVA-specific CTL responses in vivo, compared with splenocytes or RMA-S cells pulsed with OVA peptide, or compared with immunization with free OVA peptide mixed with adjuvant. Antibody-mediated depletion experiments have shown that the cytotoxic effector cells consist primarily of CD8+ cells, and that induction of CTL in vivo is dependent on CD4+ as well as on CD8+ T cells. These results provide the basis for exploring the role of bone marrow–generated DC in major histocompatibility class I–restricted immune responses, and they provide the rationale for using bone marrow–generated DC in CTL-mediated immunotherapy of cancer and infectious diseases.

The dendritic cell (DC) network is a specialized system for presenting Ag to naive or quiescent T cells and consequently plays a central role in the induction of T cell, as well as B cell, immunity in vivo (1). Several studies have documented the exceptional ability of DC to stimulate naive T cells, both in vitro and in vivo. DC are potent stimulators of primary MLR in mice and in humans (2, 3), and they are capable of sensitizing in situ CD4+ T cells to specific Ag in an MHC-restricted manner (4, 5). DC also stimulate in vitro the proliferation of allogeneic CD8+ T cells (6, 7) and the generation of Ag-specific CTL from naive precursors (8–10). Inoculation of mice with small numbers of allogeneic DC (11), or with DC pulsed with peptide (12) or whole protein (13) or transfected with DNA (14), induces strong CTL responses in vivo.

Since DC are present in trace amounts, enrichment protocols based on their low buoyancy, nonadherence to plastic, and the absence of certain cell surface markers were used to obtain sufficient cells for biological studies. However, the procedures involved were tedious and time consuming, and they generated relatively low numbers of DC. Recently, Inaba et al. (15) have developed a protocol to generate large numbers of DC from myeloid progenitors present in the bone marrow of mice, and they have shown that the bone marrow–derived DC were capable of presenting mycobacterial Ags to unprimed CD4+ T cells in vivo (16). However, the activation of CD8+ cytotoxic T cells from naive precursors by bone marrow–generated DC has not been yet reported.

Our aim was to test whether bone marrow–generated DC are capable of inducing specific CTL against tumor cells expressing a defined epitope. In this study we show that bone marrow–generated DC pulsed with an MHC class I–restricted peptide are potent inducers of specific CTL against tumor cells expressing the protein containing the CTL epitope.

Materials and Methods

Mice. 5–6-wk-old female C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were used at 7–10 wk of age.

Cell Lines and Cell Cultures. The tumor cell lines used were EL-4 (C57BL/6, H-2b, thymoma) and E7-OVA (EL-4 cells transfected with the chicken OVA cDNA [17]). Cells were maintained in DMEM supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. E7-
OVA cells were grown in medium containing 400 μg/ml G418 (GIBCO BRL, Gaithersburg, MD). EL-4 and E.G7-OVA were free of mycoplasma as tested by mycoplasma T.C. RNA detection kit (Jen-Probe, San Diego, CA).

**DC Generation from Bone Marrow Cultures.** The procedure used in this study was described by Inaba et al. (15), with some minor modifications. Briefly, bone marrow was flushed from the long bones of the limbs and depleted of red cells with ammonium chloride. Bone marrow cells were depleted from lymphocytes, granulocytes, and Ia§ cells using a cocktail of mAbs and rabbit complement. The mAbs were 2.43 anti-CD8, GK1.5 anti-CD4, RA3-3A1/6.1 anti-B220/CD45R, B21-2 anti-Ia (TIB 210, 207, 146, and 229, respectively; American Type Culture Collection, Rockville, MD), and RB6-8C5 anti-Gr-1 (kindly provided by DNAX Corp., Palo Alto, CA). Cells were plated in six-well culture plates (10⁶ cells/ml, 3 ml/well) in RPMI 1640 medium supplemented with 5% heat-inactivated FCS, 50 μM 2-ME, 10 mM Hepes (pH 7.4), 2 mM glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 3.3 ng/ml recombinant murine GM-CSF (kindly provided by Amgen Biologicals, Thousand Oaks, CA). At day 3 of culture, floating cells were gently removed and fresh medium was added. At day 7 of culture, nonadherent cells and loosely adherent proliferating DC aggregates were collected and replated in 60-mm petri dishes (10⁶ cells/ml, 5 ml/dish). At 9 or 10 d of culture, nonadherent cells (DC) were removed for analysis and immunizations.

**Peptides.** Peptides were synthesized by solid phase techniques and purified by reverse phase HPLC. The H-2Kb-restricted OVA peptide (amino acids [aa] 257-264, SIINFEKL; specified as ova in the figures) (18), and the H-2Kb-restricted influenza nucleoprotein peptide (aa 50-57, SDYEGRLI; specified as NPI(K) in the figures) (19), were purchased from Research Genetics (Huntsville, AL). The H-2Kb-restricted mut-1 peptide (FEQNTAQP) (20) was kindly provided by O. Mandelboim and L. Eisenbach (Weizmann Institute, Rehovot, Israel).

**CTL Induction in Mice.** On day 9 or day 10, DC (1-4 × 10⁶) were washed once in IMDM-50 μM 2-ME and resuspended in 1 ml of the same medium containing 100 μg of peptide. After 3 h of incubation at 37°C (with gentle shaking every 30 min), cells were washed twice in HBSS, and 10⁵-10⁶ cells per mouse (in 0.5 ml HBSS) were injected intravenously. Spleen cells were removed from mice 8-10 d after immunization. Splenocytes were depleted of APC in two consecutive steps: (a) Splenocytes from two spleens were suspended in 10 ml RPMI-10% FCS and placed for 90 min in a 75-cm² culture flask, and nonadherent cells were collected; and (b) nonadherent cells were further depleted of APC with rabbit complement and mAb cocktail RA3-3A1/6.1 anti-B220/CD45R, B21-2 anti-Ia, and F11d anti-HSA (TIB83, American Type Culture Collection). Alternatively, just the first step was carried out. Nontreated or treated splenocytes from primed mice (2 × 10⁶/ml) were cultured for 5 d in vitro with irradiated (200 Gy) E.G7-OVA cells (2 × 10⁶/ml) in six-well culture plates (5 ml/well). Culture medium was NCTC 109 and RPMI 1640 (1:1 vol/vol) supplemented with 10% heat-inactivated FCS, 50 μM 2-ME, 20 mM Hepes (pH 7.4), 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin.

**Cytotoxicity Assays.** After 5 d of restimulation, graded doses of viable lymphocytes were plated in 96-well V-bottomed culture plates (in triplicates) and cocultured for 4 h with europium diethylentriamine pentaacetate-labeled target cells (21). Europium diethylentriamine pentaacetate release was measured by time-resolved fluorescence (1232 Delfia Fluorometer; Wallac, Gaithersburg, MD). SEMs of triplicate cultures were <5% of the means.

**In Vivo Depletion of Immune Cell Subsets.** mAbs against CD4 + T cells (GK1.5), CD8+ T cells (2.43), and NK1.1 + cells (PK136; HB191, American Type Culture Collection) were used. Mice were injected intravenously once with 100 μl per mouse of hybridoma ascites 6 d before inoculation of DC and then three times in a series of injections every 4 d with 50-100 μl per mouse at 4-d intervals (the last injection was 2 d before harvesting of the spleens). Specific depletion was >85% as determined by flow cytometry (data not shown).

**Results and Discussion**

**Induction of OVA-specific CTL in Mice Inoculated with OVA Peptide-pulsed Bone Marrow-derived DC.** DC were generated from the bone marrow of C57BL/6 mice as described by Inaba et al. (15). Cell surface marker analysis, the stimulatory potential of the DC in primary allogeneic MLR, and morphological analysis have confirmed that >95% of the nonadherent cells present 9-10 d after initiating the bone marrow cultures consisted of DC (data not shown). The bone-derived DC were pulsed with an 8-aa-long peptide derived from chicken OVA (OVA peptide, aa 257-264), which encodes an H-2Kb-restricted epitope (18), and they were injected into mice via the tail vein. The presence of OVA-specific CTL in the spleen of immunized mice was determined after 8-10 d of in vitro stimulation with E.G7-OVA cells. E.G7-OVA cells were derived from EL-4 cells (H-2b haplotype) transfected with the chicken OVA cDNA (17). E.G7-OVA and EL-4 cells were used as targets.

In initial experiments we observed that immunization with bone marrow-generated DC induced a very high level of nonspecific lytic activity that largely obscured the presence of OVA-specific CTL. Nonspecific lysis was observed against both syngeneic and allogeneic targets, regardless of whether

![Figure 1](https://via.placeholder.com/150)
DC were or were not pulsed with peptide, and irrespective of the restimulator cells used or the NK/LAK sensitivity of the targets (Fig. 1 A and data not shown). Nevertheless, splenocytes from mice immunized with OVA peptide-pulsed DC and restimulated in vitro with E.G7-OVA cells lysed E.G7-OVA cells above the levels of non-specific lysis. Careful titration of the number of injected DC revealed that a dose of 0.5-1.0 x 10^6 peptide-pulsed DC induced high levels of specific CTL, whereas lytic background was reduced considerably (data not shown).

A parameter that could have contributed to non-specific lysis was the presence of APC in the splenocytic population used for restimulation in vitro. Since the bone marrow-generated DC were cultured 6-8 d in FCS-containing medium, it was possible that FCS components such as BSA were processed and presented by the bone marrow-generated DC, resulting in non-specific proliferation and activation of T cells. Indeed, Inaba et al. have observed that CD4^+ T cells obtained from mice injected with bone marrow-generated DC proliferated in response to BSA (16). We reasoned that the FCS epitopes were more likely to be processed and presented by potent APC in the splenocytic population, such as DC, macrophages, or B cells, rather than by the class II-negative E.G7-OVA cells used as specific restimulators. Therefore, we tested whether elimination of APC from the spleen before restimulation will reduce non-specific lytic activity. Spleens from mice immunized with 5 x 10^6 non-pulsed or OVA peptide-pulsed DC were harvested, and splenocytes were depleted from potential APC using complement and a cocktail of mAbs against B cells and monocytes/DC. As shown in Fig. 1 B, this led to a significant reduction in non-specific lysis with minimal reduction of specific lysis. Addition of IL-2 (5 U/ml) during restimulation of the depleted splenocytes had no effect on non-specific or specific lysis (data not shown).

For convenience, nonadherent splenocytes from which most of the DC and macrophages and some of the B cells were removed by adherence to plastic, instead of APC-depleted splenocytes, were used in subsequent experiments.

Carbone et al. (22) have shown that in vitro stimulation of a primary CTL response could be achieved using OVA peptide-pulsed splenocytes, although the CTL recognized and lysed target cells pulsed with OVA peptide but did not lyse cells transfected with and expressing the OVA protein. The concern was raised that certain methods of immunization will elicit only low affinity CTL that may not be capable of recognizing targets presenting Ag in a physiological manner. To analyze the target specificity of the cytotoxic cells elicited by the bone marrow-generated DC, mice were immunized with DC pulsed with either of two H-2K^b-restricted peptides, the OVA peptide or the mut-1 peptide derived from the D122 tumor cell line (20); stimulated in vitro with E.G7-OVA cells; and tested for effector functions against several targets: E.G7-OVA, OVA peptide-pulsed EL-4 cells, and EL-4 cells pulsed with either of two unrelated peptides encoding H-2K^b- and H-2K^k-restricted CTL epitopes, the mut-1 peptide and a peptide derived from the influenza virus nucleoprotein, NP(K^k) (19), respectively. As shown in Fig. 2 A, CTL in-
Immune Cells Involved in the Response to Immunization with DC. Effector cell populations induced by immunization with OVA peptide-pulsed DC and restimulated in vitro by E.G7-OVA cells were depleted from CD4⁺, CD8⁺, or NK1.1⁺-expressing cells using complement and specific Ab, and they were tested for effector cell function against E.G7-OVA targets. As shown in Fig. 3, only depletion of CD8⁺ cells from the effector cell population led to a significant reduction of nonspecific as well as specific lytic activity, indicating that CD8⁺ CTL were primarily responsible for both specific and nonspecific lysis induced by bone marrow–generated DC. As shown in Fig. 4, when cells were depleted in vivo, both CD4⁺ and CD8⁺ cells were found to be required for the induction of specific and nonspecific lytic activity, whereas NK1.1⁺-expressing cells played no role in either case.

The participation of Ag-specific MHC class II-restricted CD4⁺ T cells (Th) in the induction of MHC class I-restricted CD8⁺ CTL in vivo is well documented (23–26).

However, the 8-aa-long OVA peptide presented by the H-2Kb class I molecule is not known to and is unlikely to encode an MHC class II-restricted epitope. It is therefore tempting to speculate that FCS components present during the generation of DC from the bone marrow–derived precursors provide MHC class II-restricted epitopes that stimulate CD4⁺ T cell responses and enhance the OVA-specific CTL responses seen in these experiments. Experiments are in progress to test this hypothesis.

Comparative Analysis of CTL Induction by Peptide-pulsed DC, Splenocytes, RMA-S Cells, and Free Peptide Mixed with Adjuvant. Several studies have documented the effectiveness of mature DC-enriched preparations to induce CTL in vivo (11–14). It was therefore of interest to test how effective the bone marrow–generated DC were in inducing CTL responses compared with other methods. It was of particular interest to compare DC with RMA-S cells since it was recently shown that RMA-S cells pulsed with peptides were also very effective in inducing primary CTL responses in vivo and in vitro (20, 27, 28, and Nair, S., and E. Gilboa, manuscript in preparation). Bone marrow–generated DC, splenocytes, or RMA-S cells were pulsed with OVA peptide and injected into mice. RMA-S cells were first incubated at 28°C for 48 h before peptide pulsing, which was performed at 37°C. OVA peptide (100 µg) emulsified in IFA was injected into two mice subcutaneously. Nonadherent splenocytes were restimulated with E.G7-OVA cells, and cytotoxic activities were measured against EL-4 and E.G7-OVA target cells.

Highlighted points of the study:
- Depletion of CD8⁺ cells from the effector cell population led to a significant reduction of nonspecific as well as specific lytic activity.
- CD8⁺ cells were found to be required for the induction of specific and nonspecific lytic activity.
- CD4⁺ and CD8⁺ cells were required for the induction of MHC class I-restricted CD8⁺ CTL in vivo.
- The 8-aa-long OVA peptide presented by the H-2Kb class I molecule is unlikely to encode an MHC class II-restricted epitope.
- FCS components present during the generation of DC from bone marrow–derived precursors provide MHC class II-restricted epitopes that stimulate CD4⁺ T cell responses.
with peptide (data not shown). For immunization with free peptide, 100 µg of OVA peptide was emulsified in IFA and injected subcutaneously into mice. As shown in Fig. 5, neither splenocytes nor RMA-S cells were able to elicit a measurable CTL response, whereas administration of free peptide in adjuvant elicited a weak CTL response. In stark contrast, only the bone marrow-generated DC induced a strong CTL response. Similar results were obtained when EL-4 cells pulsed with different peptides were used as targets (data not shown). It is also noteworthy that immunization with DC, but not by other methods, induced a considerable level of nonspecific lytic activity.

In summary, bone marrow–generated DC, like their mature counterparts, are potent stimulators of CTL responses in vivo. Since CD8⁺ CTL responses play an important role in many infectious diseases and in cancer, it will be important to see whether CTL responses elicited by the bone marrow–generated DC will be able to protect animals from infectious agents or from tumors.

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