Dendritic Cells Cross-present Latency Gene Products from Epstein-Barr Virus–transformed B Cells and Expand Tumor-reactive CD8+ Killer T Cells

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Abstract
Dendritic cells (DCs) are not targets for infection by the transforming Epstein-Barr virus (EBV). To test if the adjuvant role of DCs could be harnessed against EBV latency genes by cross-presentation, DCs were allowed to process either autologous or human histocompatibility leukocyte antigen (HLA)-mismatched, transformed, B lymphocyte cell lines (LCLs) that had been subject to apoptotic or necrotic cell death. After phagocytosis of small numbers of either type of dead LCL, which lacked direct immune-stimulatory capacity, DCs could expand CD8+ T cells capable of killing LCLs that were HLA matched to the DCs. Necrotic EBV-transformed, major histocompatibility complex (MHC) class I–negative LCLs, when presented by DCs, also could elicit responses to MHC class II–negative, EBV-transformed targets that were MHC class I matched to the DCs. Part of this EBV-specific CD8+ T cell response, in both lytic and interferon γ secretion assays, was specific for the EBV nuclear antigen (EBNA)3A and latent membrane protein (LMP)2 latency antigens that are known to be expressed at low levels in transformed cells. The induced CD8+ T cells recognized targets at low doses, 1–10 nM, of peptide. Therefore, the capacity of DCs to cross-present antigens from dead cells extends to the expansion of high affinity T cells specific for viral latency antigens involved in cell transformation.

Key words: dendritic cells • Epstein-Barr virus • latency antigens • cross-presentation • cytotoxic T lymphocytes

Introduction
Peptides from infected or malignant cells can be presented on the MHC products of bone marrow–derived APCs (1). As the donor cells and APCs need not be genetically matched at the MHC, antigens effectively “cross” from one cell to another. Cross-presentation has been known for some time (2), but only recently have important mechanistic aspects become apparent. Dendritic cells (DCs) prove to be particularly efficient in cross-presenting antigens on both MHC class I and II (3–5). For MHC class I, cross-presentation is also termed the exogenous pathway, as the presented viral or tumor peptides need not be synthesized endogenously in the APC cytoplasm. Nevertheless, transporters associated with antigen processing (TAP) seem crucial (1, 5). The sources of antigen include virus particles (6), bacteria (7), exosomes (8), immune complexes (5), and dying cells (3, 9).

Cross-presentation of dead and dying cells is of special interest in the context of tumor immunology. Tumor cells typically lack costimulatory molecules, so the processing of tumor cells can lead to the display of multiple cancer antigens on potent DCs, improving immunogenicity. At this time, cross-priming during virus infection has been studied with infectious viruses like influenza and vaccinia (1, 3), rather than oncogenic viruses in latency or nonlytic states. In other words, the significance of cross-presentation would be greatly enhanced if one could demonstrate that latent antigens are processed, and that the corresponding primed T cells recognize epitopes naturally presented on intact transformed cells. For EBV, latency states are associated with transformation, so cross-presentation provides a way to elicit immunity to EBV antigens expressed in tumors like Hodgkin’s lymphoma and nasopharyngeal carcinoma. Here we describe the cross-presentation by human DCs of antigens from dying B lymphocyte cell lines (LCLs) trans-
formed with EBV. We will show that human DCs cross-present apoptotic and necrotic LCLs, giving rise to CD8+ effectors that directly recognize HLA-matched, transformed B cell lines, including such important latency gene products as (EBV nuclear antigen) EBNA3A and latent membrane protein (LMP)2. These findings provide a rationale for the testing of DCs loaded with dead tumor cells to elicit immu

tivity to EBV-associated malignancies in vivo.

Materials and Methods

**EBV-transformed LCLs.** LCLs were derived from PBMCs of serologically MHC class I–typed donors, using the supernatant of the marmoset line B95-8 added in the presence of 1 μg/ml cyclosporin A in RPMI 1640/20% FCS/glutamine/gentamicin. In addition, LCL721.221 (10) and T2 (11) cells were used. All established cell lines were grown in RPMI 1640/10% FCS/glutamine/gentamicin.

**DCs and T Cells.** As described (12), PBMCs from leukocyte concentrates (buffy coats) or whole blood were isolated on Ficoll-Paque (Amersham Pharmacia Biotech) and separated by ro

setting into T cell enriched (ER+) and depleted (ER-) populations. DCs were generated in 3-ml cultures of 2.5 × 10⁶ ER- cells with 1% human single donor plasma, recombinant human (rh)GM-CSF and rhIL-4 in a standard way (12) for 6 d, where

upon nonadherent immature DCs were transferred to new plates at 3 × 10⁶ cells/ml. Half the medium was replaced by monocyte-conditioned medium (MCM) as a maturation stimulus. After 48 h, mature DCs were used fresh or after cryopreservation to stimulate T cells at a ratio of 1:30 in RPMI 1640/5% human se-



**Induction and Detection of Apoptosis and Necrosis in LCLs.** Ap-

optosis was induced with a 60-mJ UV-B light (UVB) lamp (Derma Control, Inc.), calibrated to provide 2 mJ/cm²/s, and necrosis either by four freeze–thaw cycles or heating at 56°C for 30 min. Cell death was monitored using the Early Apoptosis Detection kit (Kayima Biomedical) to stain with annexin V-FITC (early apoptosis) and PI (primary or secondary necrosis).

**DC Phagocytosis of Apoptotic or Necrotic Cells.** 8 h after LCLs

were induced to undergo apoptosis or necrosis, immature DCs were added at a ratio of 1:1. DCs were matured by adding MCM. To separate DCs from the LCLs for the experiments represented by Fig. 4, b and c, DCs were stained with PE-anti-CD11c (Becton Dickinson) and isolated with anti-PE MicroBeads, MS+/RS+ column, and MiniMACS separator. More than 95% of the beaded cells were CD11c-PE+, CD83-FITC+, and CD20-FTTC-. To follow phagocytosis, LCLs were dyed red with PKH26 before inducing cell death and immature DCs green with PKH67 (Sigma-Aldrich). After coculture for 12 h at a ratio of 1:1 at 4°C and 37°C, phagocytosis was monitored as double-positive cells on the FACS®, or by applying DCs to alcan blue-coated slides for two-color fluorescence microscopy using an Olympus epifluorescence microscope. The motorized stage allowed to take 0.5-mm optical sections with a cooled CCD camera (Hamamatsu) and Metamorph software (Universal Imaging Corp.). Images were deconvoluted by applying a nearest neighbor algorithm provided by Metamorph.

**Vaccinia Virus Recombinants and Infection of DCs.** Recombi-
nant vaccinia viruses (vv) were expanded on adherent rabbit RK13 cells and titrated on monkey BSC40 kidney cells. Vv was added to mature DCs at a multiplicity of infection (MOI) of 2:1 for 1 h at 37°C and washed three times with 5% pooled human serum (PHS). The rate of infection was checked after 6–12 h by FACS® using intracellular staining for a 29-kD vaccinia early protein with VV1-6B6 antibody (12).

**Peptide Loading of DCs.** The synthetic peptides FLRGRAYGL (HLA-B8+/EBNA 3A) and CLGGGLTMV (HLA-A2/LMP 2) were purchased from Genemed Synthesis or Research Genetics, and added to APCs and targets at 1 μM in RPMI 1640 for 1 h at 37°C.

**T Cell Assays.** IFN-γ enzyme-linked immunospot (ELISPOT) and 4-h Na²⁵CrO₄ cytolysis assays were carried out in a standard way as described (12).

**Results**

**DCs Phagocytose Apoptotic or Necrotic LCLs.** We first studied DC phagocytosis of apoptotic and necrotic LCLs.
Apoptosis was induced by UV-B, and necrosis by either four cycles of freeze–thawing or heating at 56°C for 30 min. LCL death was monitored at several times using the FACS® and staining for annexin V–FITC and PI. Annexin V single-positive LCLs (apoptosis) predominated 6 h after UV-B, and annexin V/PI double positives (necrosis) after repeated freeze–thaw cycles (Fig. 1 A). Freeze–thawing killed most cells, but live cells remained after UV-B treatment (Fig. 1 A). To follow phagocytosis, the LCLs were labeled with PKH26 before induction of cell death, and 8 h later, immature PKH67-labeled DCs were added at a ratio of 1:1. After 12 h of DC/LCL coculture (for maximal uptake), ~50% of the DCs stained double positive (Fig. 1 B). Some DCs might not have taken up dying LCLs, because immature DCs generated with GM-CSF and IL-4 contained an already mature, less phagocytic fraction. No double-positive DCs were seen in cocultures on ice (Fig. 1 B). Phagocytosis of LCL fragments by DCs was verified by fluorescence microscopy (Fig. 1 C), whereas LCLs were incapable of endocytosing LCL fragments under the same conditions (data not shown). Therefore, ~50% of cultured DCs took up dying apoptotic or necrotic LCLs within 12 h.

Cross-presenting DCs Induce CTLs that Lyse LCLs HLA-matched to the DCs. To formally establish presentation across the MHC, we cocultured immature DCs with dead HLA-mismatched LCLs (HLA typing in figure legends) for 2 d and tested if the DCs would expand T cells that could kill LCLs HLA-matched to the DCs. To mature the DCs, we added MCM along with the dying cells. As expected, MCM induced a mature phenotype, i.e., CD83, CD25, and high CD86 and HLA-DR. The DC phenotype was similar whether or not they had fed on dying apoptotic or necrotic cells (not shown). The DCs then were used to stimulate ER+ T cells for 14 d, with one restimulation on day 7 with IL-2. HLA-B8+ DCs that had taken up HLA-B8+ LCLs elicited CTLs that killed HLA-B8–matched, but not HLA-B8–mismatched, LCLs (Fig. 2 A). Necrotic and apoptotic LCL fragments were similarly active as sources of LCL antigens (Fig. 2 A). DCs or dead LCLs by themselves were incapable of stimulating T cells; in fact, mixtures of DCs and dead HLA-mismatched LCLs did not induce lysis of the HLA-mismatched LCLs used for the coculture (Fig. 2 A). This indicates that apoptotic and necrotic cellular fragments lack either sufficient native HLA molecules and/
or costimulatory molecules to evoke direct alloreactivity. In addition, alloreactivity mediated through MHC-derived peptides processed from LCL was not formally required to induce lytic T cells, as shown by the use of select LCLs in the experiments of Fig. 2 B. Here, HLA-A2+ DCs were cocultured with necrotic LCL721.221 cells that lacked MHC class Ia products. The T cells expanded by the DCs were nevertheless capable of lysing MHC class II T2 cells that were EBV+ and HLA-A2+. These cultures also showed only background reactivity against PHA blasts autologous to the DCs. As T2 cells are TAP-deficient, the lysis in Fig. 2 B is probably due to recognition of EBV peptides presented in a TAP-independent manner as described for the LMP2<sub>425–434</sub> epitope (13) and formally established below in Fig. 4 C. Accordingly, T cells stimulated with LMP2<sub>425–434</sub> on DCs were used as a positive control, and untreated DCs as a negative control in Fig. 2 B. Therefore, even when exposed to low doses of dead allogeneic LCLs, DCs can cross-present EBV antigens that ultimately are presented by other LCLs HLA-matched to the DCs.

**DCs Cross-present Apoptotic or Necrotic, Allogeneic, and Autologous LCLs to CD8<sup>+</sup> T Cells.** To verify that the lysis of LCLs in Fig. 2 was mediated by CD8<sup>+</sup> T cells, we again fed DCs with apoptotic or necrotic LCLs during their maturation, but then added CD8<sup>+</sup> or CD4<sup>+</sup> selected T cells for 14 d.

**Figure 3.** CD8<sup>+</sup> T cells are effectors for HLA class I–matched LCLs after cross-presentation by DCs. (A) HLA-A2<sup>+</sup>B8<sup>+</sup> DCs were cultured with allogeneic HLA-A2-B8<sup>+</sup> LCLs (HLA-A*0101 homozygous, B*4002, B*0801, C*0304, C*0701, DRB1*0301, DRB1*1101, DQB1*0201, and DQB1*0301) at a ratio of 1:1 during maturation. The DCs were added to CD8<sup>+</sup> or CD4<sup>+</sup> positively selected T cells for 14 d. Lytic activity (4 h 51Cr release assay) is shown on HLA-A2<sup>+</sup>B8<sup>+</sup> LCLs, at E/T ratios of 20:1. (B) HLA-A2<sup>+</sup>B8<sup>+</sup> DCs, after loading with autologous necrotic or apoptotic LCLs for 2 d in MCM, were added to CD8<sup>+</sup> T cells (ratio 1:30). On day 7, wells were split for ELISPOT assays to detect IFN-γ upon restimulation with autologous vs. allogeneic HLA-A2-B8<sup>+</sup> LCLs. Necrotic LCLs alone did not expand LCL–specific T cells. One of two similar experiments. SFC, spot-forming cell; apop, apoptotic; necr, necrotic.

**Figure 4.** The EBV latent antigens EBNA3A and LMP2 are cross-presented by DCs on MHC class I. (A) HLA-A2<sup>+</sup> DCs were allowed to phagocytose allogeneic, apoptotic (UVB), or necrotic (freeze–thaw [FT]) LCLs during maturation. The DCs were cultured with syngeneic T cells, and 7 d later, ELISPOT assays were run with control recombinant vaccinia (vvTK<sup>+</sup>), vvLMP1, or vvLMP2. SFC, spot-forming cell. (B) HLA-A2<sup>+</sup>B8<sup>+</sup> DCs were cultured with apoptotic (UVB) or necrotic (FT) HLA-A2<sup>+</sup>B8<sup>+</sup> LCLs for 2 d in the presence of a maturation stimulus. Then the DCs were stained with anti–CD11c-PE, isolated with magnetic anti-PE beads, and cocultured with ER<sup>+</sup> T cells at a ratio of 1:30. 7 d later, IFN-γ–secreting T cells were quantified after no restimulation or restimulation with EBNA3A<sub>325–333</sub> peptide. (C) HLA-A2<sup>+</sup> DCs were charged for 2 d with necrotic (heated) HLA-A2<sup>+</sup> allogeneic LCLs or with LMP2<sub>425–434</sub> peptide, and simultaneously matured with MCM. Mature CD11c<sup>+</sup>PE<sup>+</sup> cells were selected with anti-PE beads and used to stimulate autologous CD8<sup>+</sup> T cells. After day 14, T cells were tested in an IFN-γ ELISPOT assay with LMP2<sub>425–434</sub> Peptide. One of three similar experiments.
The DCs and target LCLs were not HLA-DR matched (Fig 3, legend), so one would not expect CD4+ CTLs as occurs with responses to EBNA1 (4). In fact, cross-presentation expanded CD8+ T cells that lysed class I–matched LCLs, whereas CD4+ T cells showed only background lysis against the HLA class II–mismatched targets (Fig. 3 A).

We then verified that DCs expand CD8+ T cells after uptake of autologous, necrotic, or apoptotic LCLs (Fig. 3 B). These T cells secreted IFN-γ (ELISPOTs) in response to the autologous LCLs, but not to HLA-A2/B8–mismatched LCLs. Dead autologous LCLs alone did not expand specific CD8+ T cells whether the LCLs were killed via necrosis (Fig. 3 B) or apoptosis (not shown). Therefore, DCs are able to process allogeneic or autologous LCLs, directly stimulating CD8+ T cells reactive with LCLs matched at HLA class I loci to the DCs.

DCs Cross-present Defined EBV Latency Gene Products from Dying LCLs. We next tested for cross-presentation of known EBV latency gene products. DCs charged with a low dose of dead LCLs were used to stimulate purified CD8+ T cells for 1 wk. In Fig. 4 A, cross-presentation of HLA-A2– LCLs allowed HLA-A2+ DCs to expand CD8+ T cells specific for the LMP2 EBV latency antigen, i.e., HLA-A2+ DC targets were recognized by the cultures after infection with vvLMP2 but not vv control (vvTK–), or vvLMP1. To extend these experiments, we purified the DCs (see Materials and Methods) away from residual dying LCLs before the DC-T cell coculture. We used anti–CD11c magnetic bead selection, as CD11c is abundant on DCs but not detectable on LCLs. The purified DCs from HLA-B8+ and/or HLA-A2+ donors processed the dominant known latency T cell epitopes from EBV RNAs from allogeneic LCLs, specifically EBNA3A325–333 (restricted by HLA-B8; Fig. 4 B) and LMP2426–434 (restricted by HLA-A2; Fig. 4 C).

High Functional Affinity of CD8+ T Cells Expanded by DCs Cross-presenting LCLs. We used the ELISPOT assay to compare the functional affinity of CD8+ T cells expanded by DCs pulsed with viral peptide or cross-presenting necrotic LCLs. After a week of DC-T cell coculture, we added graded doses of peptide directly to the assay, avoiding further addition of APCs that increased antigen-independent backgrounds. Both forms of DCs expanded CD8+ T cells of comparable epitope affinity, as indicated by titrations of FLGRAYGL peptide (Fig. 5). Half-maximal IFN-γ responses occurred at 1–10 nM peptide (Fig. 5). Therefore, DCs extract EBNA3A peptide from latently infected LCLs, and the ensuing cross-presentation generates CD8+ T cells with a high functional affinity.

Discussion

DCs efficiently cross-present dying virus-infected cells (3). We now find that DCs also cross-present virus-transformed B cells. This extends the exogenous pathway for MHC class I in several directions: to the processing of B cells, to viral antigens expressed during latency, and to antigens (EBNA3A, LMP2) that are expressed endogenously and are likely to be critical for oncogenesis. Importantly, the cross-presentation we have studied does not require enhanced antigen expression by productive infection, exogenous interferons, or viral vectors and promoters. Also, transformed LCLs are direct targets for cross-primed CD8+ T cells. These findings have potential impact along two lines, to design new immune therapies for EBV-associated malignancies, and to understand resistance of most healthy carriers of EBV to transformation in vivo.

If one considers many EBV-associated malignancies, such as Hodgkin’s lymphoma and nasopharyngeal carcinoma, only a group of EBV latency genes are known to be expressed, i.e., EBNA1, LMP1, and LMP2 (14). Two of these can be presented through the exogenous pathway by DCs: EBNA1 on MHC class II (4) and in this paper, LMP2 on MHC class I. Furthermore, LCLs serve as targets for these CD4+ (4) and CD8+ (this paper; Fig. 3) T cells. Therefore, the cross-presenting ability of DCs, or the specific loading of DCs with CD4+ T cell (EBNA1) and CD8+ T cell (LMP2) antigens, might be explored in DC-mediated active immunization against EBV-associated malignancy. By charging DCs with necrotic rather than apoptotic LCLs, there will be much less carryover of viable tumor (Fig. 1 A). Importantly, by using DCs rather than tumor cells as the antigen, one can exploit the distinct capacity of DCs (relative to B cells) to produce high levels of IL-12 (15) and prime efficiently in the T cell areas (16). Even a stable stock of allogeneic LCLs could be feasible for charging DCs for immunotherapy, especially in cases in which autologous LCLs or tumor are difficult to obtain. Although the DCs will likely process allogeneic peptides from the LCLs (the so-called indirect pathway of allo-MHC presentation) in addition to viral latency antigens, the former antigens may provide helper function during immunization. In our hands, allogeneic MHC molecules on dead LCLs were not directly stimulatory for T cells, presumably because allo-MHC and/or necessary costimulatory molecules did not maintain their native conformations in sufficient number on dead LCLs (Fig. 2 A). While this manuscript was under review, stimulation of LCL–specific CD8+ T cells was achieved by another group using DCs loaded with LCL lysates (17). Cross-presentation from

Figure 5. T cells, generated by DCs cross-presenting allogeneic LCLs, have a high functional affinity. Titration of FLGRAYGL peptide in the ELISPOT assay after expansion with DCs loaded with synthetic FLGRAYGL peptide or dying necrotic LCLs. SFC, spot-forming cell.
allogeneic LCLs is most likely due to processing of EBV latency-specific peptides from proteins or larger protein fragments in the dead LCLs, as small peptide epitopes have very short half-lives in cells lacking their restricting HLA molecules (18) and are therefore found in only trace amounts in allogeneic LCLs.

The two latency antigens we have investigated for cross-presentation are of special interest for defense against EBV in vivo. LMP2 is the only EBV transcript expressed by EBV+ blood memory B cells from healthy donors (19). LMP2 is thought to provide a growth signal comparable to that delivered via src-family kinases and the B cell receptor (20). LMP2 also is a major EBV latency antigen expressed in Hodgkin’s lymphoma (14), against which CD8+ T cell reactivity can be detected, at least in some HLA-haplotypes (21, 22). These features make LMP2 potentially important for the induction of protective CD8+ T cell immunity, including for DC-mediated therapy against EBV-associated malignancy. EBNA3A cross-presentation demonstrates the efficiency of the exogenous pathway in DCs. The EBNA3 proteins are expressed at lower levels than other EBV latent antigens (23), and only 1–30 MHC class I–complexed EBNA3 peptides per cell can be detected on the LCL surface (24). In our studies, we reproducibly observed cross-presentation of LMP2 and EBNA3A when we loaded just one dying LCL per DC.

We suspect that cross-presentation can begin in vivo during the lytic acute phase of EBV infection (infectious mononucleosis), when the number of dying infected B cells must be massive. The immune response to EBNA1, which is essential for the maintenance of the EBV episome during host cell division, is providing the first evidence for cross-presentation of EBV antigens to CD4+ and CD8+ T cells in vivo. The Gly-Ala repeat region of EBNA1 inactivates the proteasome in cis, blocking its presentation on MHC class I by the endogenous pathway (25). However, EBNA1–specific CD8+ T cells are known, and these require exogenous loading of MHC class I molecules for presentation (26, 27). We have found that DCs also process EBNA1 from transformed LCLs onto MHC class II, expanding EBNA1–specific CD8+ T cells (4). This may occur in vivo, as the CD4+ T cell response in healthy carriers is primarily of the Th1 type (28). As B cells are not known to be efficient in producing IL-12 or polarizing T cells to Th1, whereas DCs are efficient in both regards, IL-12–producing, cross-presenting DCs may induce these EBNA1–specific Th1-type CD4+ T cells in vivo. Therefore, we suggest that the immune response to EBV may depend substantially on the cross-presentation of EBV-infected cells by DCs.

This work was supported by grants from the Cancer Research Institute and National Institutes of Health (AI40874) to R.M. Steinman, and by fellowship grants from the Cure of Lymphoma Foundation to M. Subklewe and the Lymphoma Research Foundation of America to C. Münn.

Submitted: 3 August 2000
Revised: 20 November 2000
Accepted: 1 December 2000

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