Dendritic cells efficiently cross-prime HLA class I-restricted cytolytic T lymphocytes when pulsed with both apoptotic and necrotic cells but not with soluble cell-derived lysates

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Abstract

Dendritic cells (DC) have been shown to efficiently present antigen to CD8⁺ cytolytic T cells (CTL) when pulsed with apoptotic cells as a source of cell-derived antigen. Such cross-priming could not be detected by the use of necrotic cells, while conflicting results have been reported for cell-derived soluble lysates. In this study, we reinvestigated this issue by using autologous Epstein–Barr virus-transformed lymphoblastoid cell lines (LCL) as a source of antigen to pulse monocyte-derived DC. Both autologous and HLA-mismatched allogeneic LCL have been used either in the form of apoptotic or of necrotic cells or of soluble cell lysates. At day 7, DC were co-cultured with the autologous CD8⁺ lymphocyte fraction for an additional 9 days, in the presence of exogenous IL-2 (added after 48 h). At the end of the culture period, CD8⁺ CTL efficiently lysed autologous LCL only when they had been co-cultured with DC pulsed with necrotic or apoptotic cells. That an efficient cross-priming of autologous CD8⁺ cells could be induced by DC pulsed with apoptotic or necrotic LCL but not with cell lysates was further demonstrated in assays of IFN-γ production in response to short-term re-stimulation of CD8⁺ cells with LCL. In addition, LCL-specific CD8⁺ cells could specifically lyse autologous DC that had been pulsed with LCL-derived antigens, further suggesting that DC presented exogenous antigens on HLA class I molecules.

Introduction

Dendritic cells (DC) function as a potent natural adjuvant that can currently be employed to evoke an efficient immune response (1–5). Recently, it has been demonstrated that DC can present exogenous antigens to T cells not only in the context of HLA class II, but also of HLA class I molecules, a phenomenon termed cross-priming (6–8). This could confer to DC a central role in cell-mediated immunoresponse. In a number of studies, DC have been pulsed with heterogeneous sources of cell antigens, including whole cells, total cell lysate or soluble, cell-derived, proteins (3,9,10). However, controversial data have been reported regarding the most effective approach for DC pulsing. For example, an efficient cross-priming of antigen-specific cytotoxic T lymphocyte (CTL) precursors has been primarily observed by the use of apoptotic bodies derived from influenza-virus infected DC (9). Using this approach, it has been clearly demonstrated that exogenous antigens can be presented to CTL in the context of HLA class I molecules on DC. However, apoptotic cell death may not be the only source of antigens for DC pulsing, e.g. cytopathic viruses primarily induce necrosis of infected cells. Therefore, it is conceivable that effective mechanisms must exist to provide suitable sources of antigen for effective T cell

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responses also under these conditions. Along this line, it has been reported that exogenous particulate antigens, such as ovalbumin (OVA) coupled to latex particles, was processed by H-2Kb macrophages and presented on Kb molecules up to 1000-fold more efficiently than was soluble OVA (11,12). In addition, both apoptotic (13) and necrotic cells (14) may induce DC maturation, although recent data would provide a different interpretation to at least some of these results (15). In this study, we established an autologous model employing Epstein–Barr virus-transformed B cell lines for DC pulsing. B-EBV lymphoblastoid cell line (LCL) may represent a particularly interesting model. Thus, although they carry an integrated B-EBV genome and express viral antigens, they display certain characteristics of tumor cells (16). We show that both apoptotic and necrotic LCL allow DC to exert an efficient cross-priming, leading to precursor CTL activation, proliferation and induction of effector functions. Soluble antigens derived from the same cells did not allow an efficient presentation to CTL.

Methods

Isolation of DC precursors and DC cultures

Highly purified monocytes were isolated as previously described (17) from peripheral blood mononuclear cells (PBMC) of a donor characterized by a high titer of anti-EBV antibodies. PBMC were plated at 2.5×10⁶ cells/well in 24-well plates (Costar, Cambridge, MA) in RPMI 1640 (Euroclone, Milan, Italy) without serum at 37°C in the presence of 5% CO₂. After 1 h, non-adherent cells were removed by extensive washing. To check the purity of the monocyte populations, adherent cells were detached from a single well by incubation in ice-cold PBS (Sigma, Milan, Italy) supplemented with 0.02% EDTA (Euroclone), then counted and analyzed for CD14 surface expression.

In order to obtain a population of DC capable of efficient antigen uptake, monocytes were cultured for 7 days in RPMI 1640 medium containing 5% autologous serum, penicillin and streptomycin (respectively 100 U/ml and 100 µg/ml), L-glutamine (2 mM) (Gibco, Grand Island, NY), human recombinant granulocyte macrophage colony stimulating factor (GM-CSF, 25 ng/ml), and IL-4 (100 IU/ml; Euroclone). In some experiments DC cells were further treated with tumor necrosis factor (TNF)-α (25 ng/ml; Euroclone) and lipopolysaccharide (LPS, 100 ng/ml; Sigma) in order to induce a full DC maturation.

Source of cell-derived antigens

Autologous and allogeneic LCL were established as described (18), expanded and used as a source of antigen. HLA typing was performed serologically on PBMC according to the standard National Institutes of Health micro-lymphototoxicity technique (19). The LCL used for autologous experiments were HLA A1-29, B12-w21, CW3, while for allogeneic control a A3, B7(51), CW3 LCL was used. The latter was also employed for autologous LCL DC pulsing.

Apoptotic cells were obtained by γ irradiation (50 Gy) as previously described (20). Following irradiation, cells were cultured for additional 48 h and then apoptosis was evaluated by microscopy, flow cytometric DNA analysis and Annexin V cell-surface binding (21,22).

Necrotic cells were obtained by four cycles of freezing in liquid nitrogen and thawing at room temperature. The resulting suspension, containing only cell fragments, was centrifuged for 10 min in a microfuge at 7000 r.p.m. Pellets were used as source of necrotic cells while supernatants were collected, total protein content evaluated by the BioRad protein assay (BioRad, Milan, Italy) and used as soluble antigen.

For DC pulsing, 5×10⁵ DC were resuspended in 0.5 ml of medium containing the following sources of LCL antigens: (i) soluble antigen (protein content 100 µg/ml), (ii) apoptotic cells (obtained as described above from 2.5×10⁶ irradiated LCL) and (iii) necrotic cells (obtained as described from 2.5×10⁶ LCL). Pulsing was carried out for 4 h at room temperature.

Surface molecule analysis

Analysis of cell surface markers on live cells was performed using the following mAb in immunofluorescence assays: phycoerythrin (PE)-conjugated anti-CD86 (HA5.287, IgG2b), FITC-conjugated anti-CD80 (MAB104, IgG1), PE-conjugated anti-CD1a (BL6, IgG1) and FITC-conjugated anti-CD83 (HB15A, IgG2b) (all from Immunotech, Marseille, France). Anti-HLA-DR (D1.12, IgG2a) was kindly provided by R. Accolla (HB15A, IgG2b) (all from Immunotech, Marseille, France). Anti-HLA-DR (D1.12, IgG2a) was kindly provided by R. Accolla and anti-CD14 (63D3, IgG1) by D. Vercelli, (HSR-DIBIT, Milan).

The direct immunofluorescence procedure was performed by diluting fluorochrome-labeled mAb with 1 mg/ml human γ-globulin (human therapy grade from a commercial source), in order to block non-specific Fc receptor binding. Cells were then washed and flow cytometric analysis was performed. Indirect immunofluorescence assays were performed as follows: cell-aspecific binding sites were saturated with human γ-globulin and then the relevant mAb was added and incubated for 30 min at 4°C. After extensive washing, FITC-conjugated isotype-specific goat anti-mouse antibodies (Southern Biotechnology Associates, Birmingham, AL) were added and incubated for 30 min at 4°C. Negative controls included directly labeled or unlabeled isotype-matched irrelevant mAb. Flow cytometry was performed by a FACSort (Becton Dickinson, Milan, Italy). Analysis were carried out on gated live cells using PC Lysys software (Becton Dickinson).

The Annexin V–FITC kit (Boehringer Ingelheim, Heidelberg, Germany) was employed to evaluate the number of apoptotic cells (21). Briefly, aliquots of cells were collected, washed in PBS, resuspended in the binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM CaCl₂) and mixed with 5 µl of Annexin V–FITC/10⁵ cells. After 10 min of incubation at room temperature, cells were washed, resuspended in the binding buffer containing 1 µg/ml of propidium iodide (PI) and finally analyzed using flow cytometry. The percentage of Annexin V⁺ cells was calculated on the PI⁻ cell population.

Isolation and antigen-specific stimulation of CD8⁺ T cells

Autologous CD8⁺ T cells were isolated from PBMC by first eliminating plastic adherent cells, and then cells reacting with anti-CD4, anti-CD16, anti-CD19, anti-CD14 and anti-HLA-DR mAb using goat anti-mouse IgG-coated beads and immunomagnetic depletion (Immunotech, Marseille, France). Resulting populations contained >80% of CD8⁺ cells. The
CD8^+ fraction contained <3% of CD4^+ cells and <10% of CD16^− cells, while CD3^+ CD4^+ CD8^− γδ^+ T cells were <5%. CD8^+ -enriched T cells were cultured in a 24-well Costar plate in the presence of DC pulsed with the different source of LCL-derived antigens. T cells (10^6) were co-cultured for 9 days with 10^5 DC in complete medium supplemented with 5% autologous serum. rIL-2 (20 IU/ml) was added at day 2 of culture. At day 9, T cells were harvested and analyzed for CTL specificity. LCL-specific CD8^+ T cells were further re-stimulated with specific antigen and expanded for 15 days.

**Cytotoxicity tests**

To evaluate the specific cytolytic activity of cultured CD8^+ T cells, we used as target cells autologous LCL and HLA-mismatched allogeneic LCL. Cells were labeled with 100 mCi ^51^Cr as described (22) and assays performed in triplicate using different E:T ratios.

In another set of experiments, autologous DC, either pulsed with necrotic LCL (as described above) or un pulsed, were employed as target cells for CD8-mediated cytotoxicity. The E:T ratio used was 20:1. Cytolytic assays were performed at different time intervals after DC pulsing as indicated. TNF-α and LPS were added to DC after the 4 h antigen pulsing. Unpulsed DC used as control were cultured under the same conditions.

**Intracellular cytokine detection**

Intracellular IFN-γ production is considered a suitable marker of specific CD8^+ T cell activation. For this reason, T cell populations obtained by different sources of antigen were collected and re-stimulated with autologous or allogeneic LCL for 5 h at 1:10 stimulator:responder ratio, in the presence of brefeldin (5 μg/ml). T cells were then tested for surface CD8 expression and intracellular IFN-γ production using direct immunofluorescence and flow cytometry. To this end, after staining with FITC-labeled anti-CD8 mAb (Becton Dickinson), cells were washed in PBS, then fixed in 4% formaldehyde for 5 min on ice, washed in PBS and permeabilized by incubation in PBS containing 0.1% saponin. Non-specific mAb binding was blocked by saturation with human γ-globulin (0.5 mg/ml) diluted in saponin for 10 min. PE-labeled anti-IFN-γ mAb (PharMingen, San Diego, CA) was subsequently added (0.5 μg/10^6 cells in 0.1% saponin) and incubation was carried out for 30 min. Cells were washed twice in permeabilization buffer and the expression of relevant molecules was analyzed on the FACSort by electronically gating on lymphocytes, excluding debris and large aggregates.

**Results**

**Both apoptotic and necrotic LCL induce an efficient DC-mediated cross-priming of specific CTL**

DC were obtained from peripheral blood-derived CD14^-monocytes in the presence of GM-CSF and IL-4. Cultured cells rapidly lost CD14, and acquired CD1a, CD40, CD80 and CD86, as previously described (17,23). These surface markers were homogeneously expressed by all cultured cells at day 7. After pulsing with different LCL-derived antigens, including apoptotic or necrotic cells or soluble antigen, day 7 DC were co-cultured for 9 days with CD8^-enriched autologous T cells and 20 IU/ml of rIL-2 (added after 48 h). Cultured T cells were then tested at day 9 for their capability to lyse autologous LCL previously used for DC pulsing. Allogeneic LCL were used as a control. Figure 1 shows the result of a representative experiment. Only T lymphocytes that had been stimulated with DC pulsed with either apoptotic or necrotic autologous LCL displayed specific CTL activity. On the other hand, soluble antigen-pulsed DC failed to induce any detectable CTL response. The same T cell populations did not lyse allogeneic HLA-mismatched LCL. Notably, the DC phenotype acquired during the 7-day differentiation period remained stable also after antigen pulsing and no differences were detected among the three different pulsing conditions (not shown).

**CTL specific for autologous LCL produce IFN-γ in response to specific re-stimulation**

To further analyze the specificity of CTL responses, the intracellular production of IFN-γ was evaluated using immunofluorescence and flow cytometry after 5 h re-stimulation with autologous LCL. Figure 2 shows the results of a representative experiment. IFN-γ production could be detected in CD8^+ cells re-stimulated with autologous, but not with allogeneic LCL. Only CD8^+ cells primed with DC that had been pulsed with either necrotic or apoptotic cells were efficiently stimulated, whereas soluble antigens were ineffective. To confirm specificity for autologous LCL of primed CD8^+ T cells, they were further cultured with autologous LCL for 15 days. During this additional culture interval, cells underwent extensive proliferation. Moreover, as shown in Fig. 2, after this additional culture interval, >30% of CD8^+ T cells produced IFN-γ in response to re-stimulation with autologous LCL (Fig. 2, BOOST). Note that although the soluble antigens were insufficient to induce specific responses by CD8^+ cells (no CD8^+ cell producing IFN-γ could be detected), they did induce responses in the CD8^- cell fraction. This CD8^- cell fraction...
CD8⁺ cross-priming by DC pulsed with necrotic or apoptotic cells

**Fig. 2.** IFN-γ production by cross-primed CD8⁺ cells in response to specific re-stimulation. CD8⁺ lymphocytes that had been co-cultured with autologous DC pulsed with different sources of B-EBV LCL antigens were re-stimulated with autologous or allogeneic LCL. Horizontal axis: expression of CD8 surface molecule. Vertical axis: in the upper panel, staining with isotypic IgG control after autologous B-EBV LCL re-stimulation; in the lower panels, intracellular IFN-γ induced by re-stimulation with autologous or allogeneic B-EBV LCL. CD8⁺ T cells were cultured with DC pulsed with autologous apoptotic LCL (APOP), necrotic LCL (NECR) and soluble antigens (LYS). Unpulsed DC (DC) were used as controls. BOOST, intracellular IFN-γ production in CD8⁺ T cells primed by necrotic cell pulsed DC, cultured for additional 15 days with autologous LCL and then re-stimulated as described above.

Evidence that LCL-specific CD8⁺ cells selectively lyse DC pulsed with LCL-derived antigen(s)

These experiments were designed to investigate whether LCL-specific CD8⁺ cells could indeed recognize LCL-derived antigens on mature DC. This information would provide evidence that CD8⁺ priming was mediated by antigen recognition on DC. To this end, DC that had been pulsed with LCL-derived antigen(s) were further allowed to differentiate into mature DC (high expression of CD83, CD80 and CD86, not shown) by culture with TNF-α and LPS (24), and then used as target cells for LCL-specific CD8⁺ cells. Figure 3 shows that CD8⁺ cells efficiently lysed pulsed mature DC but had no effect on unpulsed mature DC. Moreover, it indicates that DC are susceptible to lysis only after a relatively short time interval after antigen pulsing.

Necrotic allogeneic LCL can induce DC-mediated cross-priming of specific CTL

These experiments were performed under the same conditions described in Methods. As shown in Fig. 4 (left panel), DC pulsed with necrotic cells, but not with cell lysate or apoptotic cells, efficiently cross-prime autologous CD8⁺ cells, which were able to lyse autologous LCL.

CTL derived from culture with autologous DC pulsed with allogeneic apoptotic cells were unable to lyse autologous LCL, despite the fact that they underwent strong activation and proliferation during culture. This is likely to reflect the occurrence of a strong alloreactive response towards apoptotic cells, which, differently from necrotic cells, partially maintain their own shape and structures. Highly proliferating alloreactive cells (mostly represented by CD4⁺ cells) would dilute the LCL-specific CTL present in the culture. Indeed, CTL derived from cultures containing DC pulsed with apoptotic cells showed a strong cytolytic activity when tested
against the same LCL employed as source of antigen (Fig. 4, right panel).

Discussion

In the present study, we provide evidence that both necrotic and apoptotic autologous LCL may represent a suitable source of antigens for DC-mediated priming of CD8\(^+\) precursor CTL. On the other hand, soluble antigens derived from the same LCL were ineffective.

Previous reports suggested that only apoptotic cells functioned as a soluble source of antigens for CD8\(^+\) lymphocyte cross-priming (8,9). Our data indicated that both apoptotic and necrotic cells may be used to pulse DC and to generate efficient CD8\(^+\) CTL responses. The specificity of the CD8\(^+\) cell-mediated responses was indicated by the selective lysis of autologous, but not of unrelated LCL. Likewise IFN-\(\gamma\) production was only induced upon re-stimulation with autologous LCL. These data rule out the possibility of a potential CTL activation via non-specific mechanisms (e.g. NK-like activity). Further evidence of the specificity was obtained by the selective proliferation of specific CD8\(^+\) cells, upon re-stimulation with autologous LCL. Interestingly, if T cells, co-cultured with DC that had been pulsed with necrotic cells, were further cultured for an additional 15 days in the presence of antigen, the percentage of IFN-\(\gamma\)-producing CD8\(^+\) T cells increased \(\geq\)6-fold.

That priming of CD8\(^+\) cells during culture was indeed mediated by DC presenting LCL antigen(s) was strongly suggested by the ability of primed CD8\(^+\) cells to lyse DC pulsed with autologous LCL. The results of these experiments also imply that CD8\(^+\) mediated lysis is consequent to a HLA class I-restricted antigen recognition.

In these experiments, the possibility that necrotic cell pulsing could induce DC maturation (14) thus modifying their susceptibility to lysis (25–27) is unlikely. Thus, a full DC maturation was obtained in both pulsed and unpulsed DC, because of the addition of TNF-\(\alpha\) and LPS in both cultures. Indeed DC obtained in these experiments homogeneously express high levels of CD83, HLA-DR, CD80 and CD86.

A recent report demonstrated that DC, during phagocytosis of dying cells, would acquire intact membrane proteins, including foreign HLA molecules. The newly acquired HLA would be fully functional because it allows recognition by allospecific T cells, and the binding and presentation of antigen peptides. As a consequence, DC become ‘immunological hybrid’ as they display their own and foreign HLA molecules (28). In order to investigate the possible role of this phenomena in our study, we performed DC pulsing also with allogeneic HLA-mismatched LCL. Also under these conditions, a recruitment of LCL-specific CD8\(^+\) cells by DC pulsed with allogeneic necrotic cells could be detected. This ruled out the possibility that DC could prime CD8\(^+\) cells only because they displayed the HLA class I molecules of LCL on their surface. This also supported the notion that DC pulsed with necrotic cell-derived antigens are able to process the acquired antigen and present the related peptides on HLA class I molecules in order to cross-prime CD8\(^+\) lymphocytes.

Remarkably, the use of soluble antigens (derived from the same LCL) failed to induce any detectable CD8\(^+\)-specific response even when used at high protein concentration. On the other hand, these soluble antigens appeared to preferentially induce CD4\(^+\) cell responses as suggested by the IFN-\(\gamma\) production by these cells after specific re-stimulation.

After phagocytosis, different alternate HLA class I processing mechanisms are possible: exogenous antigens could be digested in vacuola and peptides, without reaching the cytosol, could bind HLA class I molecules within post-Golgi vacuolar compartments or directly at the cell surface after ‘regurgitation’ from intracellular processing compartments. In contrast, antigen fragments could escape from vacuolar compartments into the cytosol, where they would undergo cytosolic processing, transport by TAP and binding of antigenic peptides to HLA class I in the endoplasmic reticulum. The striking efficiency of particulate antigens, such as necrotic or apoptotic cells, compared to soluble antigens, suggests mechanistic hypotheses. For example, the level of antigen uptake that can be achieved by a population of phagocytic cells could be higher with a particulate antigen. In addition, in the course of phagocytosis a large amount of DC cell membrane is internalized. This may provide surface HLA class I molecules with direct access to phagocytic processing compartments, where they could bind peptides derived from exogenous phagocyted antigens, if peptides show high affinity and are concentrated enough. Thus, considering that most of the cell membrane internalized during phagocytosis is recycled, the new peptide–HLA I complexes could be eventually expressed on the DC surface for presentation to CD8\(^+\) cells. According to this model, the nature of the antigens and the affinity and concentration of the related peptides would be critical. This could also explain why other authors did not observe DC-mediated cross-priming employing necrotic cells as a source of exogenous antigens (9). Indeed, EBV LCL is likely to carry a strong antigenic repertoire, which might allow phagocyted necrotic cells to be presented by this pathway. Using other antigens, only apoptotic cells, probably more effective in the cross-priming phenomena because of specific surface markers (29–31) or
other yet unclarified factors (heat shock proteins?), would be able to induce CD8+ T cell priming.

On the contrary, the possibility that particulate antigens could induce a different degree of maturation and thus mediate a greater activation of the CD8+ effectors can be ruled out by the finding that DC pulsed with different antigen sources displayed a similar surface phenotype.

The ability of necrotic cells, and not exclusively apoptotic cells, to induce an efficient cross-priming may not be surprising. Thus, cell necrosis is frequently associated to infections with life-threatening cytopathic viruses, against which a rapid CTL response is strictly needed.

Taken together, our present data may be useful for a rational development of novel strategies of vaccination against infectious diseases and cancer. For example, it would be of interest to investigate whether similar results may be obtained by using autologous EBV+ B cell lymphomas or nasopharyngeal carcinomas as a source of antigens for DC-mediated vaccination.

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CD8+ cross-priming by DC pulsed with necrotic or apoptotic cells


