MHC class II-mediated apoptosis of mature dendritic cells proceeds by activation of the protein kinase C-delta isoenzyme

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Abstract

The mature dendritic cell (DC) is considered to be the most potent antigen-presenting cell. Regulation of the DC, particularly its survival, is therefore critical. Mature DC are markedly more sensitive to HLA-DR-mediated apoptosis than immature DC. To further characterize this key survival difference, we compared the intracellular signals initiated via HLA-DR in mature versus immature DC. Apoptosis was unchanged by inhibition of tyrosine kinases or phosphatases. HLA-DR-mediated re-localization of protein kinase C (PKC)-δ to the nucleus was detected in mature DC by confocal microscopy and by immunoblotting. Activation of PKC-δ in mature DC was revealed by the detection of the PKC-δ catalytic fragment in the nuclear fraction isolated from mature DC which had been stimulated via HLA-DR. The broad-spectrum PKC inhibitor, Calphostin C, as well as the PKC-δ-selective inhibitor, Rottlerin, inhibited HLA-DR-mediated apoptosis of mature cells. Taken together, these data reveal a role for the PKC-δ isoenzyme in regulating HLA class II-mediated apoptosis of mature DC. Thus, the lifespan of the mature DC could be controlled by signals generated in the course of antigen presentation, and thereby prevent DC persistence and prolonged stimulation of T and B lymphocytes.

Introduction

HLA class II molecules are constitutively expressed in professional antigen-presenting cells (APC) of dendritic/monocytic and lymphoid lineages. Successful presentation of peptide antigens via HLA class II antigens is a requisite for the generation of T_h cell-mediated immune responses. Dendritic cells (DC) are considered the most potent APC (1). DC present in peripheral tissues are immature, and are characterized by their high efficiency of internalization and processing. Maturation takes place in the presence of inflammatory cytokines in the course of migration from the tissues to peripheral lymph nodes within which the mature DC can carry out its specialized role of antigen presentation. DC can be generated by cultivating monocytes in the presence of granulocyte macrophage colony stimulating factor (GM-CSF) and IL-4; addition of tumor necrosis factor (TNF)-α leads to maturation (2). A model is therefore available for the study of DC throughout their differentiation and maturation.

In addition to their role in antigen presentation, signal transduction via MHC class II molecules has been widely documented and leads to diverse consequences for the MHC class II-expressing cell (3). Recent studies in B lymphocytes and in monocytes have revealed the implication of Syk kinase in the production of IgM (4), and of both ERK and p38 MAP kinases in the production of the pro-inflammatory cytokine IL-1β (5). Tyrosine phosphorylation of the BCR-associated proteins CD79 α and β via MHC class II engagement was revealed in murine B lymphocytes (6). Moreover, a role for specific microdomains of the plasma membrane has been demonstrated in both signal transduction and in antigen presentation via MHC class II antigens (7–9). Both the

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signaling pathways and the consequences of signaling via MHC class II antigens vary depending on the MHC class II-expressing cell and the stimulating ligand. A clear demonstration of the importance of the form of the stimulating ligand is provided by the study of Tabata *et al*. (4). Whereas fixed HLA class II antibodies induced IgM production, neither apoptosis nor B cell proliferation was induced in the presence of soluble HLA-DR mAb. A consequence of HLA-DR-mediated signaling is apoptosis of mature B lymphocytes which proceeds by an apparently 'caspase-independent' pathway despite the observation of typical membrane and nuclear features of apoptosis (10). We have reported that whereas immature DC were relatively resistant to HLA-DR-mediated apoptosis, mature DC were susceptible to apoptosis and acquisition of susceptibility corresponded to their acquisition of markers defining mature APC (11). Since tyrosine protein kinase and PKC activation are early features of HLA-DR-mediated signals in various APC (6,12), we compared their activation via HLA-DR in both immature and mature DC.

Activation of the PKC family of serine/threonine kinases is implicated in many cellular responses including gene expression, cytoskeletal mobility, proliferation, differentiation and apoptosis (13). HLA class II signaling has diverse effects on PKC including transcriptional regulation and enzyme activation (14). Translocation of PKC-α and -βII (belonging to the classical group of PKC isoenzymes) via HLA-DR has been described (15,16). PKC-α has been described as a anti-apoptotic isoenzyme (17), whereas PKC-δ has been described as a pro-apoptotic isoenzyme (18). PKC-δ is a member of the novel group of PKC isoenzymes and does not therefore require calcium for activation. The role of PKC-δ in apoptosis is at least 2-fold because overexpression of the cleaved 40-kDa catalytic fragment of PKC-δ induced the appearance of apoptotic morphology (18) and it has also been described as a substrate for cleavage by caspase-3 (19). Phorbol ester stimulation led to mitochondrial translocation of PKC-δ and apoptosis via a cytochrome *c*-release pathway (20). There is also evidence that PKC-δ activation enhances caspase-3 activation (21). A pharmacological inhibitor which is selective for PKC-δ has been described (22).

Unlike many hematopoietic cells, mature DC do not have recourse to CD95 mediated apoptosis (23). This study examines the mechanism of an apoptotic pathway mediated by the cell surface molecules responsible for the major function of DC, i.e. antigen presentation. As such this pathway to apoptosis could permit auto-regulation of the DC lifespan.

Methods

Monomorphic HLA-DR (L243) mAb was purified from ascitic fluid. Murine IgG2a isotype control Ig was obtained from PharMingen (San Diego, CA). Fas/CD95 agonistic 7C11 mAb was purchased from Immunotech. (Marseille, France). GM-CSF, IL-4 and TNF-α were purchased from Gibco/BRL (Rockville, MD).

Safingol is a specific inhibitor of PKC-α and Rottlerin is a selective inhibitor of PKC-δ (Calbiochem, Merck Eurolab, Limonest, France). Calphostin C was from Calbiochem. Herbimycin A (3.3 μM) inhibits tyrosine kinases (Sigma, St Quentin Fallavier, France) (24). Sodium orthovadanate (1mM) is an inhibitor of protein tyrosine phosphatases (Sigma, St Louis, MO) (25).

Preparation of DC

DC were prepared from healthy donors as described (5) by culturing monocytes with 800 U/ml GM-CSF and 1000 U/ml IL-4. Half of the medium was replaced at day 3, 5 and 7 by fresh medium containing 800 U/ml GM-CSF and 500 U/ml IL-4. At day 7, 100 U/ml TNF-α was added to the culture to induce DC maturation which was confirmed by CD83 expression (11).

Localization of PKC-α and -δ by confocal microscopy

The subcellular localization of PKC isoenzymes was detected by indirect immunostaining and confocal microscopy as previously described (15,16). Cytospins were prepared with 2.5×10^5 cells stimulated with 5 μg/ml of L243 mAb or IgG2a for 20 min. We have previously detected optimal activation and translocation of PKC via HLA-DR after 20 min (14). Cytospins were fixed in cold methanol before staining with PKC isoenzyme-specific antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA). FITC-conjugated secondary mAb (The Binding Site, Birmingham, UK) were used and nuclei were counter-stained with propidium iodide. Fading of fluorescence was retarded with 1,4-diazobicyclo(2,2,2)octane (Dabco; Merck Eurolab). Immunofluorescence was detected by laser scanning confocal microscopy using a MRC 500 confocal microscope (BioRad, Marnes-la Coquette, France).

Subcellular fractionation of DC

DC (10^7) were either stimulated via HLA-DR for 5 min or left untreated before incubation in hypotonic HB buffer containing 20 mM Tris (pH 7.4), 2 mM $MgCl_2$, 10 mM EGTA, 2 mM EDTA, 2 mM DTT and a cocktail of protease inhibitors (see below). Cells were then snap-frozen in liquid nitrogen for 30 s before heating to 37°C for 2 min and mechanically lysed by homogenization. Lysates were centrifuged at 1000 *g* for 10 min at 4°C. The pellet (enriched in nuclei) was resuspended in 100 μl of HB buffer containing 1% Triton X-100 and the supernatant was centrifuged at 10,000 *g* for 10 min at 4°C. The resulting pellet (enriched in mitochondria) was resuspended in 100 μl HB-T and the supernatant was ultracentrifuged at 100,000 *g* for 1 h at 4°C. The supernatant (cytosolic fraction) was recovered as well as the insoluble cytoskeletal fraction. The quantity of protein in each fraction was determined and equal amounts of protein (20 μg) were migrated in each well; PKC-δ was detected by immunoblotting.

Detection of tyrosine phosphorylation/dephosphorylation

Tyrosine phosphorylation was detected by immunoblotting with an anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY). DC (3×10^6) were treated with 20 μg/ml of L243 or IgG2a for 2 min, washed and lysed at 4°C in lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 10 mM NaF, 10 μg/ml aprotinin, leupeptin and pepstatin, and 1 mM PMSF). Post-nuclear cell lysates were separated by SDS–PAGE (12%) under reducing conditions and transferred to nitrocellulose (Hybond ECL; Amersham, Paris, France). After blocking with PBS/0.1% Tween and 3% BSA, the membrane was incubated with 1 μg/ml 4G10 for 1 h followed by horseradish peroxidase-

conjugated secondary antibody. Tyrosine phosphorylation was revealed by chemiluminescence using ECL reagents (Amersham).

Detection of apoptosis

Annexin V binding. DC were plated in 200 μl of IMDM/10% FCS at a density of 5×10^5 cells/well, in 96-well plates, in the presence of inhibitors or an equivalent volume of inhibitor diluent (DMSO or ethanol). After a 1-h incubation, 5 μg/ml L243 or IgG2a was added and cells were stained with FITC-labeled Annexin V (Boehringer Mannheim, Meylan, France) in order to quantify apoptosis 6 h later. We have previously validated this method of detecting apoptosis in DC (11)–Annexin V binding compared favorably with both propidium iodide uptake and detection of DNA strand breaks.

Where indicated, specific apoptosis was calculated as follow: % specific apoptosis = 100 × [(% of Annexin V^+ cells in assay) – (% of Annexin V^+ cells in control)]/[100 – (% of Annexin V^+ cells in control)].

Determination of mitochondrial membrane potential ($\Delta\Psi_m$). $\Delta\Psi_m$ was evaluated by staining cells (10^6) with 3,3-dihexyloxacarbocyanine iodide ($DiOC_6$) (Molecular Probes, Eugene, OR) at a final concentration of 40 nM (stock solution 1 μM in ethanol) for 15 min at 37°C in the dark after 6 h of stimulation. The fluorescence emitted by cells was analyzed with a FACScan flow cytometer (BD Biosciences, Le Pont-du-Claix, France) using the FL-1 channel. Mature DC were pretreated for 30 min with either Rottlerin or an equivalent volume of DMSO before addition of either L243 (5 μg/ml) or the isotype control.

Immunoblotting of PKC-δ

Protein (20 μg) from each subcellular fraction was migrated in a 10% SDS–PAGE gel and transferred to Hybond PVDF membrane (Amersham). Membranes were blocked overnight at 4°C with 3% non-fat milk in PBS, 0.1% Tween and then probed with anti-PKC-δ (Santa Cruz Biotechnologies) at a dilution of 1/3000 before incubating with HRP-conjugated donkey anti-rabbit (Amersham) for 1 h at room temperature. The blot was washed and developed using ECL chemiluminescence (Amersham) according to the manufacturer's instructions.

Results

Mature DC are more susceptible to HLA-DR-mediated apoptosis than immature DC

Immunophenotyping (differential expression of CD83, CD80 and CD86) and morphology confirmed the maturation state of the DC as previously described (11). Figure 1 compares apoptosis induced via HLA-DR for 6 h in mature versus immature DC. This time was selected for the following reasons: since increased Annexin V binding was already detected at this time point, non-specific apoptosis in the presence of inhibitors was limited and because this study was concentrated on initiation events in the apoptotic pathway. Apoptosis induced by L243 was 26.2 ± 9.5% in immature DC and 46.1 ± 12.4% in mature DC (n = 5) compared with 10.8 ± 3.6% in immature DC and 19 ± 1.6% in the presence of an isotype control IgG. These results therefore concord not only with our previous data (11), but also with a recent study of murine DC in which mature DC were sensitive to MHC class II-mediated apoptosis, while immature DC were resistant (23).

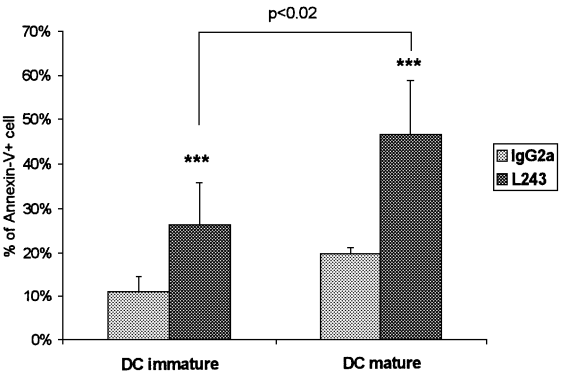

Fig. 1. Mature DC undergo apoptosis via HLA-DR. Immature and mature DC were incubated with either IgG2a or L243 for 6 h before detecting apoptosis by measuring Annexin V binding. The mean apoptosis + SD is shown (n = 5).

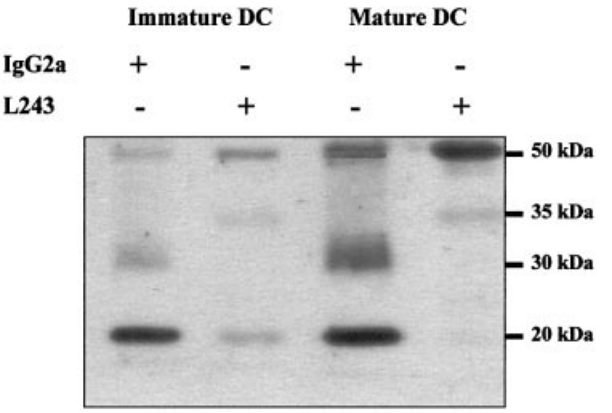

Fig. 2. Tyrosine phosphorylation and dephosphorylation is induced via HLA-DR in both immature and in mature DC. Lane 1, isotype control-treated immature DC. Lane 2, L243-treated immature DC. Lane 3, isotype control-treated mature DC. Lane 4, L243-treated mature DC. Increased phosphorylation of a 50- and 35-kDa substrate is indicated as is dephosphorylation of a 30- and 20-kDa substrate. Substrates migrating with the same mol. wt were affected in both immature and in mature DC. Representative of experiments using DC from five different donors.

Tyrosine kinases/phosphatases are activated via HLA-DR in both mature and immature DC

A sample of 25 μg of protein was migrated in all wells. Increased tyrosine phosphorylation of proteins migrating at 50 and 35 kDa was detected in immature and in mature DC (Fig. 2). Phosphorylation of the 35-kDa substrate was not detected prior to stimulation. Dephosphorylation of constitutively tyrosine-phosphorylated substrates with relative mol. wt values of 30 and of 20 kDa was observed. The 30-kDa substrate was completely dephosphorylated via HLA-DR in immature and mature DC. Importantly, substrates of identical

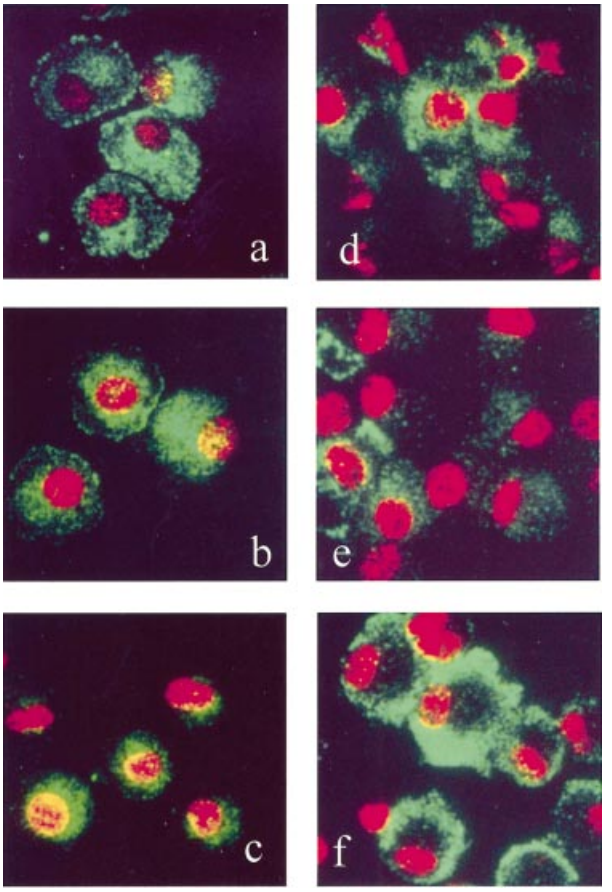

Fig. 3. Localization of PKC-α and -δ in immature DC. PKC isoenzyme immunoreactivity is indicated by FITC fluorescence (green), the nucleus was stained with propidium iodide (red). Co-localization is indicated in yellow. PKC-α and -δ were both expressed in both immature DC. (a) Diffuse, cytosolic and punctuate plasma membrane staining of PKC-α in IgG2a-treated immature DC. (b) Increased immunostaining of PKC-α in L243-treated cells, modest translocation to the plasma membrane is also observed. (c) Nuclear redistribution of PKC-α mediated by PMA treatment. (d) Cytosolic localization of PKC-δ in isotype control-stimulated immature DC. L243 treatment did not alter this distribution (e), whereas PMA treatment enhanced PKC-δ expression and particularly at the plasma membrane (f).

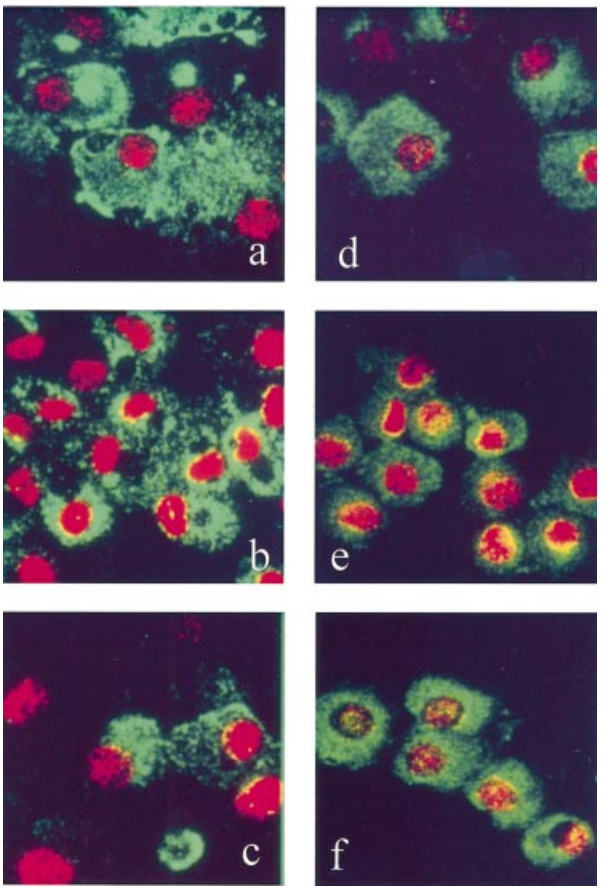

Fig. 4. Localization of PKC-α and -δ in mature DC. PKC-α staining was diffuse and predominantly cytosolic in isotype control-treated mature DC (a). Stimulation with L243 led to some perinuclear staining without a visible decrease in the cytosolic level (b). This was also the case for PMA treatment which led to some perinuclear staining without modifying cytosolic staining (c). In mature DC, PKC-δ staining was cytosolic and diffuse (d). L243 stimulation led to a striking nuclear relocalization and a corresponding decrease in the cytosolic staining (e). PMA treatment led to diffuse cytoplasmic staining and to some nuclear relocalization (f). The staining shown is representative of that detected in DC from three different donors.

mol. wt were targeted in the mature and in the immature DC (albeit to different degrees). This pattern was representative of the tyrosine phosphorylation observed via HLA-DR in DC from five different donors.

Activation of PKC isoforms via HLA-DR in DC

PKC activation is a consistent feature of MHC class II signaling in both murine and human APC (3). Since PKC activation is associated with translocation of the enzyme from the cytosolic fraction to the particulate fraction in many signaling pathways, including via HLA-DR (14), confocal microscopy provided the method of choice to examine PKC activation given the limited number of primary human DC available from a given donor. We examined PKC-α and -δ localization in immature and in mature DC before and after stimulation with L243.

PKC-α and -δ are expressed in both immature and mature DC

Figure 3(a–c) shows the distribution of the PKC-α isoform in immature DC after stimulation with IgG2a, with L243 or with the phorbol ester phorbol myristate acetate (PMA) respectively. The distribution of PKC-α was predominantly cytosolic and diffuse in IgG2a treated cells with some punctuate staining at the plasma membrane. Stimulation via HLA-DR led to a less diffuse and/or enhanced staining pattern and more continuous plasma membrane staining (Fig. 3b). PMA stimulation led to nuclear localization. In contrast to PKC-α, HLA-DR-mediated stimulation of immature DC did not perturb the cytosolic localization of PKC-δ (Fig. 3e) in comparison with isotype control-stimulated cells (Fig. 3d). PMA treatment enhanced PKC-δ expression and particularly at the plasma membrane (Fig. 3f).

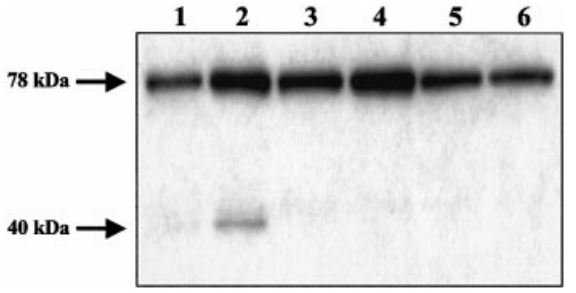

Fig. 5. Biochemical detection of nuclear translocation and activation of PKC-δ mediated via HLA-DR. Mature DC were stimulated with isotype control Ig (lanes 1, 3 and 5) or with L243 (lanes 2, 4 and 6) for 5 min at 37°C before lysing on ice, and fractionation into nuclei (1 and 2)-, mitochondria (3 and 4)- and cytosol (5 and 6)-enriched fractions. A sample of 20 μg of each fraction was migrated, transferred and blotted to detect PKC-δ. Both the full-length (78 kDa) and the cleaved catalytic fragment (40 kDa) of PKC-δ are detected by this antibody. The ratios of PKC-δ full-length form in L243 versus isotype control stimulated were as follows: nuclear 1.68, mitochondrial 1.26 and cytosolic 0.80. The catalytic fragment of PKC-δ was detected only in the L243-stimulated nuclear fraction (lane 2).

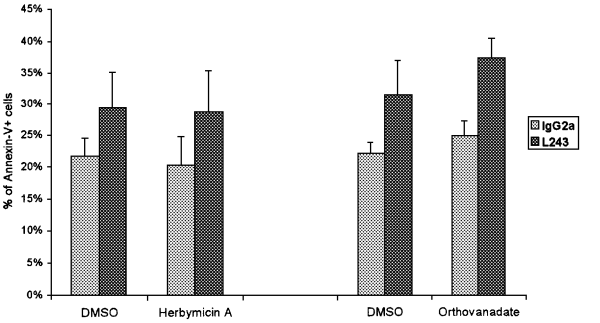

Fig. 6. Tyrosine kinase inhibition does not alter HLA-DR-mediated apoptosis of mature DC. Figure 6 illustrates the absence of inhibition of either Herbimycin A or of sodium orthovanadate on HLA-DR-mediated mature DC apoptosis. Neither inhibition of tyrosine kinases nor of tyrosine phosphatases resulted in changes in the L243-induced apoptosis ($n = 4$).

PKC-α staining was diffuse and predominant in the cytosol of isotype control-treated mature DC (Fig. 4a). Stimulation with L243 led to some perinuclear staining without a visible decrease in cytosolic staining (Fig. 4b). Likewise, PMA treatment also led to some perinuclear staining (Fig. 4c) also in the absence of modified cytosolic staining.

In mature DC, PKC-δ was cytosolic and diffuse (Fig. 4d). L243 stimulation led to a striking nuclear relocalization and a corresponding decrease in the cytosolic staining (Fig. 4e). PMA treatment led to less diffuse cytoplasmic staining and to some nuclear relocalization (Fig. 4f), albeit to a lesser degree than observed with L243. These data are representative of three experiments using DC from different individuals.

Activation and enrichment of PKC-δ in the subcellular fraction enriched in nuclei

In order to confirm the HLA-DR-driven relocalization of PKC-δ which was observed by confocal microscopy, a biochemical approach was taken (Fig. 5). Mature DC were separated into fractions enriched for nuclei (Fig. 5, lanes 1 and 2), mitochondria (Fig. 5, lanes 3 and 4) and cytosol proteins (Fig. 5, lanes 5 and 6), and immunoblotting was carried out to detect PKC-δ. After stimulation of mature DC via HLA-DR, an enrichment in the full-length PKC-δ protein (78 kDa) level was observed in the nuclear fraction. Arbitrary quantification of the bands corresponding to PKC-δ was carried out using the NIH Image-based Scion software (Scioncorp, Frederick, MD) and the ratio of the full-length form of PKC-δ in L243 (Fig. 5, lanes 2, 4 and 6)- compared to isotype control (Fig. 5, lanes 1, 3 and 5)-stimulated DC was as follows: nuclear fraction 1.68, mitochondrial fraction 1.26 and cytosolic fraction 0.80. The increases detected in the nuclear-enriched and, to a lesser degree, the mitochondria-enriched fractions were therefore paralleled by a decrease in the cytosolic fraction.

Most importantly, the catalytic fragment of PKC-δ (40 kDa) was detected in the nuclear fraction of the L243-stimulated DC (Fig. 5, lane 2), but not in either the nuclear fraction of isotype control-stimulated DC (Fig. 5, lane 1) nor in any other fraction, demonstrating that activated PKC-δ was specifically relocalized to the nucleus via HLA-DR-mediated stimulation. PKC-δ proteolysis by caspase-3 and nuclear translocation has been detected during spontaneous apoptosis of neutrophils, and was inhibited by Rottlerin (18). Further, overexpression of the 40-kDa catalytic fragment of PKC-δ has been associated with induction of apoptotic morphology (19).

Tyrosine kinase/phosphatase activation is not required for HLA-DR-mediated death of mature DC

Having observed tyrosine phosphorylation via HLA-DR, we tested whether or not this was necessary for apoptosis of mature DC. Herbimycin A inhibits activation of tyrosine kinases (24), but failed to have any inhibitory effect on the induction of HLA-DR-mediated cell death (Fig. 6). L243 induced specific apoptosis in 37.3 ± 16.5%, pre-treatment and incubation with Herbimycin A did not significantly alter this response (32.1 ± 8.9%). Apoptosis of immature DC was not affected by the presence of Herbimycin A (data not shown).

We also examined the effect of Na_3VO_4, a broad-spectrum inhibitor of protein tyrosine phosphatases (25), since the immunoblots had revealed tyrosine dephosphorylation. Na_3VO_4 failed to alter HLA-DR-mediated apoptosis in mature DC, L243 alone induced apoptosis in 40.1 ± 14.7%; in the presence of orthovanadate, L243 induced apoptosis 45.6 ± 14.7%. Taken together, the activation of tyrosine kinases/phosphatases in mature DC via HLA-DR is unlikely to have a crucial role in HLA-DR-mediated apoptosis.

HLA-DR-mediated apoptosis of mature DC is inhibited by Calphostin C

Having observed relocalization of the PKC-δ isoform via HLA-DR in mature DC, we first examined the effect of the highly specific PKC inhibitor Calphostin C on HLA-DR-mediated apoptosis. The inhibitory action of Calphostin C is due to its binding of the regulatory domain of PKC. Inhibition of specific apoptosis was observed in a dose-dependent fashion with almost total inhibition at a concentration of 50 nM, which corresponds well with the reported IC_{50} for PKC (Fig. 7A,

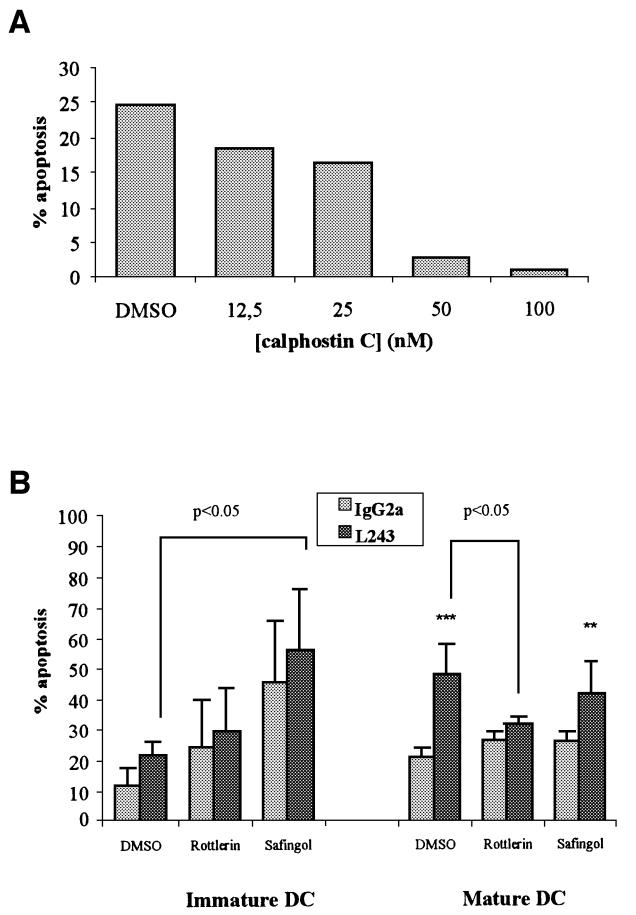

Fig. 7. PKC inhibition blocks HLA-DR-mediated apoptosis of mature DC. Selective inhibition of PKC-δ activation inhibits HLA-DR-mediated apoptosis. (A) Inhibition of HLA-DR-mediated apoptosis by Calphostin C. Specific apoptosis is shown (L243 in comparison with isotype control) and the concentration of Calphostin C is indicated (nM). Results from one of two donors examined. (B) Comparison of L243-induced apoptosis in the presence of PKC isoenzyme-specific inhibitors Safingol or Rottlerin (15 μM or DMSO) in immature versus mature DC. Neither Safingol nor Rottlerin specifically inhibited L243-mediated apoptosis in immature DC (n = 4), although Safingol increased non-specific apoptosis. Whereas Rottlerin specifically inhibited L243-mediated apoptosis in mature DC, Safingol was without effect (n = 5).

background apoptosis did not exceed 12% under all conditions tested).

HLA-DR-mediated apoptosis of mature DC requires activation of PKC-δ but not of PKC-α

Given the inhibition of HLA-DR-mediated apoptosis by Calphostin C and given the nuclear translocation of PKC-δ, we went on to examine the effect of PKC isoenzyme-selective inhibitors. Safingol is a specific inhibitor of PKC-α (26) and does not affect other PKC isoforms while Rottlerin selectively inhibits PKC-δ (27). The reported IC_{50} of Rottlerin for PKC-δ is 3–6 μM, whereas it is 30 μM for PKC-α. Safingol significantly increased the level of background apoptosis in immature DC, but failed to have any specific effect on HLA-DR-mediated apoptosis (Fig 7B: IgG2a and DMSO 11.6 ± 6.3%, IgG2a and Safingol 44.9 ± 21.1%, L243 and Safingol 55.8 ± 20.5%). The increased background apoptosis in the presence of Safingol and the punctuate distribution of PKC-α at the plasma membrane of IgG2a-stimulated immature DC supports the notion of PKC-α as an 'anti-apoptotic' isoform (13,17).

When mature DC were examined, Safingol did not change HLA-DR-mediated apoptosis (Fig. 7B, specific apoptosis was determined as: IgG2a and DMSO 20.6 ± 3.3%, IgG2a and Safingol 25.3 ± 3.8%, L243 and Safingol 40.9 ± 11.5%, L243 and DMSO 47.6 ± 10.6%). On the contrary, addition of Rottlerin significantly decreased apoptosis of mature DC induced via HLA-DR without altering non-specific apoptosis. Apoptosis induced via HLA-DR was 47.6 ± 10% and was reduced to 31.1 ± 3% in the presence of Rottlerin ($P < 0.05$). The difference between the PKC activation via HLA-DR in immature versus mature DC was further shown by the absence of effect of Rottlerin in immature DC (Fig. 7B, L243 and Rottlerin 29 ± 14.7%, L243 and DMSO 21.4 ± 5%).

Table 1.

Stimulation	Inhibitor	Apoptosis (%)
L243	DMSO	61
L243	Rottlerin 2.5 μM	34
L243	Rottlerin 5 μM	19
IgG	DMSO	5
IgG	Rottlerin 2.5 μM	4
IgG	Rottlerin 5 μM	9

HLA-DR-mediated depolarization of the mitochondrial membrane is decreased by PKC-δ inhibition

In order to confirm the inhibition of HLA-DR-mediated apoptosis as a result of inhibition of PKC-δ, we examined another characteristic of HLA-DR-mediated apoptosis, that of mitochondrial membrane depolarization. The data shown in Table 1 reveal that the number of mature DC undergoing a decrease in their $\Delta\Psi_m$ 6 h after addition of L243 mAb to mature DC was markedly decreased when the experiment was carried out in the presence of Rottlerin.

Discussion

DC survival is crucial in order to permit optimal antigen presentation at the appropriate site.

The initiating step of HLA class II-mediated antigen presentation is specific TCR-mediated engagement of the appropriate HLA class II molecule, engagement of HLA-DR by specific mAb provides a model for studying the signals transmitted via HLA class II in the course of antigen presentation. The L243 mAb was used in this study since it binds in immediate proximity to the peptide binding site of the DR heterodimer (28).

We and others have previously reported sensitivity of mature DC to apoptosis via HLA class II, whereas immature DC are relatively insensitive (11,23). We have examined the early signaling events mediated via HLA-DR in mature versus immature DC in order to identify events implicated in the

strikingly different apoptotic response of mature versus immature DC to HLA-DR stimulation.

We report that HLA-DR-mediated signals distinguished immature and mature DC since nuclear relocalization of PKC-δ only occurred in mature DC. Three complementary approaches were taken to study the localization and activation of PKC-δ: confocal microscopy, immunoblotting to detect the full-length form and the catalytic fragment, and inhibition of PKC-δ activation by both a broad-spectrum PKC and an isoenzyme selective pharmacological inhibitor.

Tyrosine kinase/phosphatase activation was observed in both mature and immature DC, but does not appear to be implicated in the HLA-DR pathway leading to mature DC apoptosis.

HLA class II-mediated signaling in DC has been reported in studies which did not address the question of apoptosis. For example, Kushnir *et al*. (29) reported HLA-DR-mediated heterotypic aggregation of DC with B lymphocytes. A critical role for PKC-α, -β and -μ isoenzymes (30) has been previously reported in a human myelomonocytic cell line model of DC differentiation. We observed tyrosine phosphorylation/dephosphorylation events in immature and in mature DC, but found no effect of inhibition of tyrosine kinases or phosphatases on HLA-DR-induced apoptosis. HLA-DR-mediated signaling in DC has been previously reported to induce tyrosine kinase activation which was necessary for expression of IL-1β mRNA (31). Moreover, in B lymphocytes, HLA-DR-mediated tyrosine phosphorylation has been implicated in activation rather than apoptosis (32).

The role of mitogen-activated protein kinase (MAPK) activation in MHC class II-mediated apoptosis has not been explored, although differential activation of ERK and of p38 MAPK has been reported via HLA-DR, -DP and -DQ, and an equilibrium between the activation of various MAPK is important in determining whether a cell survives or undergoes apoptosis (33). B-chronic lymphocytic leukemia cells undergo apoptosis via a CD20-mediated pathway of $p38^{MAPK}$ activation (34), transformed B cell lines undergo p38 dependent BCR-mediated apoptosis (35) and both of these cell types are sensitive to HLA-DR-mediated apoptosis.

Previous studies of signaling motifs, in both the mouse (36) and in the human (16), revealed that tyrosine kinase and PKC activation were mediated by different regions in the MHC class II β chain. A role for PKC activation in HLA-DR-mediated death was suggested by the inhibition of HLA-DR-mediated apoptosis of B lymphocytes in the presence of broad-spectrum PKC inhibitors (32). The current study in DC reveals a specific role for the PKC-δ isoenzyme in HLA-DR-mediated apoptosis in mature DC. The importance of PKC-δ activation in HLA-DR-mediated apoptosis of mature DC was confirmed by the inhibition noted in the presence of the broad-spectrum PKC inhibitor Calphostin C and particularly in the presence of Rottlerin. Two complementary approaches were taken to determine apoptosis—Annexin V binding, which detects apoptosis at the level of the plasma membrane, and $DiOC_6$ fluorescence, which specifically determines apoptosis at the level of the mitochondria on the basis of $\Delta\Psi_m$. A previous study reported inhibition of activation of PKC-δ with Rottlerin at a final concentration of 15 μM in monocytes (22), we have used the same conditions to inhibit apoptosis of monocyte-derived DC.

Previous studies in hematopoietic cells have revealed PKC-δ as a pro-apoptotic isoform, and include spontaneous neutrophil apoptosis (18), Fas-induced apoptosis of T cells (37) and ionizing radiation-induced apoptosis of myeloid leukemia cells (38). At least two nuclear targets of PKC-δ have been identified—DNA-dependent protein kinase (DNA-PK) (39) and lamin B (40). Interaction of DNA-PK with PKC-δ leads to inhibition of its DNA repair function (39) and PKC-δ phosphorylation of lamin B is required for lamina disassembly.

Engagement of HLA-DR by mAb as ligands is unlikely to mimic the full range of events initiated in the course of the T cell interaction with an APC. Nonetheless, the TCR–MHC class II interaction is an indispensable and initiating step in the peptide-specific interaction. In this respect, it is crucial to note that Matsue *et al*. (41) revealed apoptosis of DC after interaction with an antigen-specific T_h cell clone, whereas apoptosis was not observed when DC were cultured with either peptide or with T cells alone.

In summary, activation of PKC-δ has been demonstrated as a determining step in the apoptosis of different hematopoietic cells (19,37,38), and these data therefore support the notion that HLA-DR-mediated signals in mature DC integrate a common and potent apoptotic pathway. These data could therefore be important for the design of studies of therapeutic uses of mature DC.

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Abbreviations

$\Delta\Psi_m$	mitochondrial transmembrane potential
APC	antigen-presenting cell
DC	dendritic cell
$DiOC_6$	3,3-dihexyloxacarbocyanine iodide
DNA-PK	DNA-dependent protein kinase
GM-CSF	granulocyte macrophage colony stimulating factor
HRP	horseradish peroxidase
MAPK	mitogen-activated protein kinase
PKC	protein kinase C
PMA	phorbol myristate acetate
TPK	tyrosine protein kinase
TNF	tumor necrosis factor

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