MHC class II signaling function is regulated during maturation of plasmacytoid dendritic cells

Bernard Drénou,* Laurence Amiot,* Niclas Setterblad,† Sophie Taque,* Valérie Guilloux,* Dominique Charron,† Renée Fauchet,* and Nuala Mooney†,[1]

Laboratoire d'Hématologie et de Biologie des cellules sanguines (UPRES 22-33), Faculté de Médecine, Rennes, Cedex, France; and †Immunogénétique Humaine, INSERM U396, Institut Universitaire d'Hématologie, Paris, Cedex, France

Abstract: Dendritic cells (DC) play a central role in the immune response, linking innate and adaptative responses to pathogens. Myeloid DC (MDC) produce interleukin-12 in response to bacterial stimuli, whereas plasmacytoid DC (PDC) produce high levels of type I interferon upon viral infection. Human leukocyte antigen (HLA)-DR engagement has been shown to induce apoptosis in various antigen-presenting cells (APC). We now report the consequences of HLA-DR molecule engagement in human PDC, which had thus far not been studied as a result of the difficulty in isolating such cells. HLA-DR engagement on PDC, obtained using a two-step, immunomagnetic separation, led to recruitment of HLA-DR molecules at the site of engagement in mature but not immature PDC. In contrast, relocalization of protein kinase C (PKC) isoenzymes, indicating PKC activation, was observed at the site of HLA-DR engagement and was accompanied by relocalization of a lipid raft marker, the ganglioside M1 staining, in immature and mature PDC. Similar to MDC, HLA-DR-mediated apoptosis was regulated throughout PDC maturation. Freshly isolated PDC were resistant, whereas CD40 ligand-matured PDC were sensitive to HLA-DR-mediated apoptosis. Neither caspase activation nor PKC activation was required for HLA-DR-mediated apoptosis. However, the intrinsic pathway of apoptosis was implicated as mature PDC underwent mitochondrial depolarization in response to HLA-DR engagement. These data provide further arguments for considering HLA-DR-mediated apoptosis as a conserved mechanism of regulating survival of diverse APC and support the ongoing development of humanized ligands for HLA class II molecules as therapeutic tools for use in lymphoproliferative disease. *J. Leukoc. Biol.* 77: 560–567; 2005.

Key Words: *major histocompatibility complex · apoptosis · immune synapse*

INTRODUCTION

Dendritic cells (DC) are the most potent antigen-presenting cells (APC), which play a key role in the initiation and the regulation of the immune system [1]. DC are a heterogeneous bone marrow-derived population, as two distinct differentiation pathways can be initiated from hematopoietic CD34+ stem cells leading to myeloid [2] or lymphoid cells [3]. Myeloid DC (MDC/DC1) have been characterized extensively; they can be differentiated from myeloid precursors, express myeloid antigens, and require granulocyte macrophage-colony stimulating factor for their survival [4]. In human peripheral blood, immature MDC are identified as $CD11c^+$/human leukocyte antigen (HLA)-DR^+ cells [5] and do not express lymphoid lineage markers. These immature cells can differentiate to become interstitial DC or Langerhans cells, depending on the microenvironment. In response to inflammatory stimuli, MDC differentiate into mature DC, expressing high levels of the costimulatory molecules CD40, CD80, and CD86 and producing high levels of interleukin (IL)-12 [6], which in turn, contributes to the polarization of naive T lymphocytes toward the T helper cell type 1 (Th1) cytokine secretion profile [7].

Immature lymphoid DC or plasmacytoid DC (PDC/DC2) have been identified in lymphoid tissues (human tonsils, spleen, and thymus) as an extremely minor population of human peripheral blood mononuclear cells (~0.3%). These cells have not been characterized extensively in human, first, as a result of the difficulty in obtaining sufficient numbers of cells and second, as a result of the lack of specific markers. PDC differ from MDC in the following respects: They have a plasmacytoid morphology; they are identified in human peripheral blood as Lin^-/$CD11c^-$/HLA-DR^+/$CD4^+$low/CD45 receptor A^+/IL-3 receptor α^+ cells [8] and also express the recently described blood DC antigen (BDCA)-2 and BDCA-4 (neuropilin) antigens [9]; PDC express high levels of the pre-T cell receptor (TCR) α (pTα) and immunoglobulin (Ig)λ-like transcripts [10]; PDC are IL-3-dependent for their survival and differentiation, and their maturation can be induced via CD40 ligand (CD40L) [8]; activated PDC can induce T cell polarization [11]; in response to viral infection, they represent the major cellular source of type I interferon (IFN) [12]. PDC thus participate in the generation of antiviral and proinflammatory responses as well as T lymphocyte Th2 polarization.

[1]Correspondence: INSERM U396, Institut Universitaire d'Hématologie, Hôpital Saint-Louis, 1 av. Claude Vellefaux, 75475 Paris, Cedex 10, France. E-mail: nuala.mooney@histo.chu-stlouis.fr

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Finally, recent studies have led to the characterization of malignant proliferations of PDC, identified as leukemic PDC, which strongly resemble their normal counterpart in terms of their capacity to produce IFN-α and to behave as APC [13–15].

HLA class II molecules are constitutively expressed in professional APC, such as MDC and B lymphocytes. The profile of major histocompatibility complex (MHC) class II molecule expression has been used to characterize the maturation state of the MDC, as expression is largely intracellular in immature MDC, whereas maturation of MDC leads to transport of MHC class II molecules to the cell surface. We [16] and others [17] have documented the localization of MHC class II molecules in lipid-rich microdomains, termed "lipid rafts". The relevance of the lipid raft localization of MHC class II molecules has been described in antigen presentation [18, 37, 41] and in signal transduction [19]. Maturation of MDC also regulates the function of the MHC class II molecules; cell-surface MHC class II of immature DC is rapidly internalized following ligand-binding, whereas this capacity for internalization is lost by DC maturation. Finally, in addition to the phenotypic and functional changes in MHC class II throughout MDC maturation, signal transduction via the MHC class II molecules is also modified by APC maturation, as mature DC become sensitive to MHC class II-mediated apoptosis in the course of maturation [20, 21]. This is likely to represent a major regulatory pathway of apoptosis, as mature DC are resistant to apoptosis mediated via the death receptor Fas [20].

Apoptosis of APC by monoclonal antibodies (mAb) binding to MHC class II molecules has been documented extensively (reviewed in refs. [22, 23]). The physiological relevance of this apoptosis of mature/activated MHC class II-expressing cells is likely to be in the homeostasis of the adaptive immune response by actively removing such cells, thereby preventing further T cell activation. MHC class II-mediated peptide presentation to a peptide-specific T cell clone leads to apoptosis of a major proportion of APC of B lymphocyte or DC origin [24, 25]. It is interesting that this physiological response to MHC class II molecule-binding by a specific ligand is now under study as a therapeutic tool. We initially revealed the sensitivity of primary B lymphoproliferations to HLA class II-induced apoptosis by a seemingly caspase-independent apoptotic pathway [26]. The recent in vivo study by Nagy et al. [27] not only confirmed and extended these data but also revealed that humanized HLA class II-specific mAb strongly inhibited proliferation of human B cell lymphomas without having a lasting, detrimental effect on haematopoiesis.

The mechanisms leading to HLA-DR-mediated apoptosis are as yet unclear. Whereas mAb-driven apoptosis follows a Fas- and caspase-independent pathway, apoptosis of APC following peptide presentation via HLA-DR involved a Fas-independent but caspase-dependent pathway [25], and another study in mice revealed a partial implication of the Fas pathway [24]. In contrast, the implication of the intrinsic mitochondrial pathway in HLA-DR-mediated apoptosis has been documented consistently [20, 28, 30]. Regulatory elements of the HLA-DR-mediated pathway of apoptosis, which have been identified, include the actin cytoskeleton of the APC and activation of the serine/threonine kinase family of isoenzymes, the protein kinase C (PKC) family [20, 28–30]. In contrast, CD40 ligation on the APC provides an important but short-lived counter-signal, protecting from MHC class II-induced apoptosis [20].

This study provides the first data about the HLA-DR-transduced signal in PDC, although this population plays a key role in tolerance-induction and in antiviral immunity. The aim of this work was to characterize the HLA-DR expression precisely, as well as the consequences of HLA-DR engagement throughout PDC maturation. We quantify cell-surface expression of HLA-DR and characterize the recruitment of HLA-DR, PKC, and ganglioside M1 staining (GM1) to the specific site of HLA-DR engagement at the surface of the PDC and the regulation of HLA-DR-mediated apoptosis throughout PDC maturation.

Similar to MDC, HLA-DR signaling in PDC may thus regulate the lifespan of PDC, thereby contributing to the regulation of immune responses. These data are also relevant to establish the usefulness of humanized ligands for HLA-DR molecules developed for clinical use in the relatively rare cases of PDC proliferations and the more frequently observed B lymphoid proliferations.

MATERIALS AND METHODS

Purification of PDC

PDC were isolated from apheresis products of human peripheral blood by two-step immunomagnetic sorting. First, $CD3^+$ T cells, $CD11b^+$ monocytic cells, and $CD16^+$ natural killer cells were depleted using the blood DC isolation kit [magnetic cell sorter (MACS), Miltenyi Biotec, Bergish Gladbach, Germany]. Cells were then incubated with the "PDC-specific mAb" anti-BDCA-2, and BDCA-2-positive cells were then positively selected on a magnetic column (BDCA-2 cell isolation kit, MACS, Miltenyi Biotec).

Phenotypic analysis and HLA-DR quantification of the PDC

The purity of PDC was evaluated by flow cytometry using a FACSCalibur cytometer (Becton Dickinson, San Jose, CA) by triple-staining with a cocktail of lineage mAb: Lin-fluorescein isothiocyanate (FITC), CD123-phycoerythrin, HLA-DR-peridinin chlorophyll protein (Becton Dickinson). PDC correspond to a $Lin^-/HLA\text{-}DR^+/CD123^+$ population. Intracytoplasmic CD83 expression in fully CD40-matured PDC was revealed by immunocytochemistry because of the low number of available cells. CD83 was not expressed at the cell surface. Immunocytochemical staining of PDC with an isotype-control Ig (IgG2b) was negative (data not shown).

Flow cytometric quantitative analysis of HLA-DR molecules was performed using the "quantitative immunofluorescence indirect kit" (Qifikit, Dako, Glostrup, Denmark). The procedure consisted of labeling cells with the primary anti-HLA-DR mAb (Bl2, Immunotech/BeckmanCoulter, Marseille, France) at saturating concentrations (10 μg/ml) at 4°C. An irrelevant mouse mAb was used in parallel as a negative control. Cell samples and calibration beads precoated with different defined quantities of mouse antibodies were labeled with FITC-conjugated anti-mouse secondary antibody and analyzed by flow cytometry. The mean fluorescence intensities recorded from cells and from beads are correlated with the number of bound primary antibody molecules. The results obtained using calibration beads allow the calculation of the precise number of antigenic sites present at the cell surface. A minimum threshold of 5000 sites/cell can be detected by this method.

Molecular characterization of PDC

Total RNA was extracted using the RNAble reagent (Eurobio, Courtaboeuf, France) according to the manufacturer's instructions and then reverse-transcribed using Superscript (Life Technologies, France) with random hexamer

primers. Polymerase chain reaction (PCR) assays were performed as described previously [10] for 35 cycles at an annealing temperature of 55°C with the following primers: pTα sense 5′-GTCCAGCCCTACCCACAGGTGT-3′ and pTα antisense 5′-CCTGGCTGTAGAAGCCTCTC-3′. PCR products were examined after agarose gel electrophoresis stained with ethidium bromide.

Cell culture

Purified PDC (5×10^5 cells/ml) were cultured in medium (RPMI 1640, Invitrogen, Cergy-Pontoise, France) supplemented with 10% of fetal calf serum (FCS), 2 mM L-glutamine, antibiotics, and IL-3 (10 ng/ml; Tebu, Le-Perray-en-Yvelines, France). Fully mature PDC were prepared by providing a maturation stimulation via CD40 in IL-3-containing medium as above [CD40L (0.5 ug/ml), Alexis, Illkirch, France; and CD40L enhancer (1 ug/ml), Alexis, for 48 h]. As PDC survival is strictly IL-3-dependent, PDC were studied immediately post-isolation (for characterization and confocal microscopy) or after culture in IL-3-containing medium with or without the maturing agent CD40L as indicated (for apoptosis).

Localization of HLA-DR and PKC-α, -δ by confocal microscopy

The localization of HLA-DR or of the PKC isoenzymes was detected by immunostaining and confocal microscopy as described previously [31]. Anti-HLA-DR mAb (L243 purified from ascites) or isotype-control Ig (IgG2a, BD Biosciences, San Jose, CA) were immobilized on polystyrene beads according to the manufacturer's recommendation (Cat. #7312, Polysciences, Warrington, PA). Immature (freshly isolated) or CD40L-matured PDC (5×10^4 cells) were allowed to interact with L243-coated beads or IgG2a-coated beads for 15 min at 37°C (ratio: two beads per cell). The cells were then cytospun and air-dried for 20 min before fixation in cold methanol (PKC-α and -δ) or in 2.5% paraformaldehyde (PFA)/phosphate-buffered saline (PBS), followed by permeabilization with 0.1% Triton X-100 (for HLA-DR and GM1). PFA-fixed cells were incubated with 50 mM NH_4Cl in PBS. Cells were incubated in 1% bovine serum albumin/PBS and then washed in PBS and labeled with primary antibodies (polyclonal anti-PKC-α Ab and PKC-δ Ab, Santa Cruz Biotechnology, Santa Cruz, CA), FITC-conjugated L243 mAb, or with FITC-labeled cholera toxin (CT; Sigma Chemical Co., St. Louis, MO). PKC Ab-binding was detected with Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes, Eugene, OR). Slides were washed in PBS and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Images were acquired with the laser-scanning microscope (LSM) software on a Zeiss LSM-510 laser-scanning confocal microscope (Carl Zeiss, Oberkochen, Germany), equipped with a Zeiss Axiovert 100M (plan Apochromat 63X 1.40NA oil-immersion objective). The images shown are representative of three experiments with independent donors.

Detection of HLA-DR-mediated apoptosis in PDC

PDC were plated in 200 μl RPMI 1640 10% FCS at a density of 5×10^5 cells/ml in 96-well plates in the presence of IL-3 alone or in the presence of IL-3 and CD40L. L243 mAb or the irrelevant IgG2a isotype (5 μg/ml, BD Biosciences) was added, and after incubation for 48 h, apoptotic cells were detected by staining with FITC-labeled Annexin V (Beckman Coulter, Paris, France) and propidium iodide (PI). Apoptosis was then determined by flow cytometric determination of Annexin V-positive/PI-negative cells.

Caspase and PKC inhibitors

Caspase inhibitors Z-Asp-Glu-Val-Asp-fluoromethylketone (z-DEVD-fmk), Z-Val-Ala-Asp (z-VAD)-fmk, and z-Ile-Glu-Thr-Asp (IETL)-fmk were from France Biochem (Meudon). All are cell-permeable and irreversible; z-DEVD-fmk is selective for caspase-3, z-VAD-fmk inhibits processing of caspase-2, -3, -6, and -7, whereas z-IETD-fmk selectively inhibits caspase-8.

Calphostin C (Calbiochem, Merck Eurolab, Limomest, France) is a highly specific, broad-spectrum inhibitor of the PKC family of isoenzymes (inhibitory concentration 50%, 50 nM). CD40L-matured PDC were preincubated with Calphostin C (25, 50, and 100 nM) for 45 min before addition of L243 mAb. An equivalent volume of inhibitor diluent [dimethyl sulfoxide (DMSO) or ethanol] was used as a negative control.

Determination of mitochondrial membrane potential (ΔΨm)

ΔΨm was evaluated in CD40-matured PDC (10^5), stimulated for 6 h with the HLA-DR mAb L243 or the isotypic control IgG2a. Cells were labeled with 3,3-dihexyloxacarbocyanine iodide ($DiOC_6$; Molecular Probes) at a final concentration of 40 nM (stock solution, 50 μM in DMSO) for 15 min at 37°C in the dark. Mitochondrial membrane depolarization of CD40-matured DC was analyzed on a FACScalibur flow cytometer.

RESULTS

Morphological, phenotypic, and molecular characterization of PDC

Ex vivo isolated PDC were examined after May-Grünwald Giemsa staining and revealed typical plasma cell-like morphology (**Fig. 1A**) characterized by an excentric nucleus, a blue basophilic cytoplasm, and a pale Golgi region. Maturation induced via CD40 in IL-3-containing medium for 48 h led to acquisition of typical DC morphology. Less extension of den-

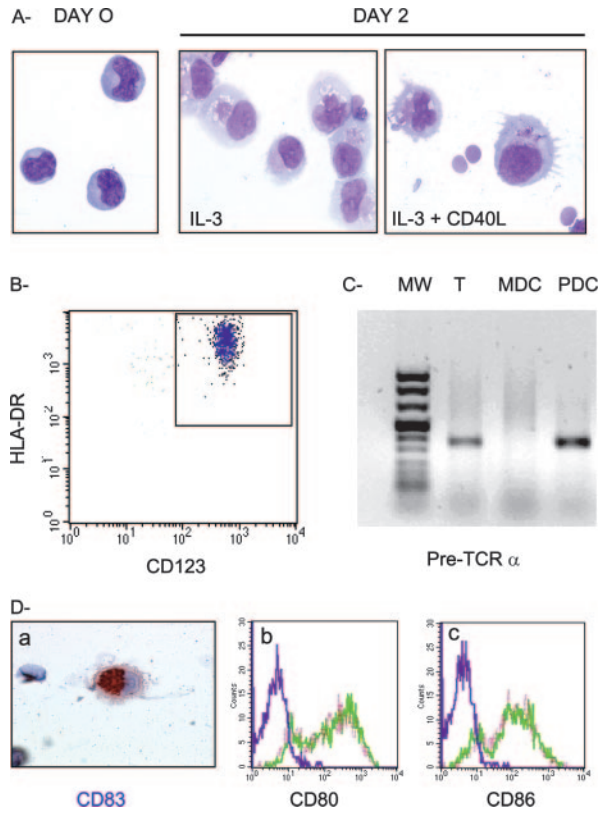

Fig. 1. Characterization of isolated PDC. (A) May-Grünwald Giemsa staining demonstrated characteristic morphology of freshly isolated PDC, more lightly stained nuclei and cytoplasm were observed after maintenance for 48 h in IL-3-containing medium, and cells that had received a maturation stimulus via CD40 had typical mature DC morphology. (B) Expression of cell-surface HLA-DR and CD123 was determined by flow cytometry in freshly isolated PDC. (C) The pTα (Pre-TCR α) transcript was examined by PCR in thymic extract (T), MDC, or PDC and was readily detected in thymic extract and PDC. MW, Molecular weight. (D, a) Intracytoplasmic CD83 was detected by immunocytochemistry in IL-3 + CD40L-derived DC (in brown); (b) PDC cultured in IL-3 containing medium in the absence (green) or presence of CD40L (pink) were tested for CD80 (b) and CD86 expression (c) and compared with freshly isolated PDC (purple).

drites was observed in cells maintained in IL-3 in the absence of the CD40-mediated maturation stimulus and is shown for comparison.

Phenotypic analysis of freshly isolated PDC shows that more than 90% of the cells are HLA-DR$^+$, CD123$^+$ (Fig. 1B), Lin$^-$, CD11c$^-$ (not shown). Reverse transcriptase-PCR studies confirmed the presence of the transcript of pTα (Fig. 1C), which is absent from MDC. Intracytoplasmic expression of CD83, a marker of DC maturation, was detected by immunocytochemistry (Fig. 1D). The same immunocytochemistry protocol was followed to label CD40-matured PDC with an isotype-control Ig for the CD83 mAb (IgG2b); no staining was detected (data not shown).

Freshly isolated, immature PDC scarcely expressed CD80 or CD86 in comparison with PDC maintained in IL-3 for 48 h (green trace) or PDC fully matured via CD40 (pink trace; Fig. 1D, b and c). Therefore, maintenance of PDC in IL-3-containing medium led to up-regulation of CD80 and CD86, although the morphology did not indicate full maturation (see Fig. 1A).

Quantification of HLA-DR on PDC

The absolute number of cell-surface HLA-DR molecules was quantified on freshly isolated, immature PDC (n=3) and on PDC matured via CD40 for 48 h (n=3). The number of HLA-DR molecules on immature PDC was evaluated at 69,500 ± 40,500 sites compared with 662,500 ± 770,000 sites on mature PDC, representing a tenfold increase in surface expression induced by PDC maturation.

Localization of HLA-DR in PDC

Freshly isolated, immature PDC were compared with CD40-matured PDC; the stimulus is indicated at the top of each column, and the protein detected is indicated on the left side of **Figure 2**. Cells were stimulated in the presence of isotype-control, Ig-coated beads (columns 1 and 3) or HLA-DR mAb-coated beads (columns 2 and 4).

Confocal microscopy revealed a rather diffuse pattern of HLA-DR expression in immature PDC, which was much more punctuate in mature PDC (Fig. 2A). Whereas HLA-DR staining was homogeneous in the immature PDC, it was almost exclusively detected at the plasma membrane and in an intracellular structure resembling the Golgi apparatus in the mature cells.

The use of mAb-coated beads to initiate signal transduction is particularly useful when limited cell numbers are available, as the site of interaction can be observed. We compared the outcome of signaling after allowing HLA-DR mAb-coated beads to interact with freshly isolated immature or mature PDC. IgG2a-coated beads were used as a negative control, and where possible, an image of a cell/bead interaction is shown. The same numbers of cells/beads were used under all test conditions, although cell/bead interactions are not shown in all cases for the isotype control-coated beads. This is due to the rarity of "stable" interactions formed between cells and IgG2a-coated beads compared with HLA-DR mAb-coated beads. Stimulation of immature PDC via HLA-DR molecules did not alter the distribution of the HLA-DR molecules in immature DC. This result contrasts with the marked recruitment of

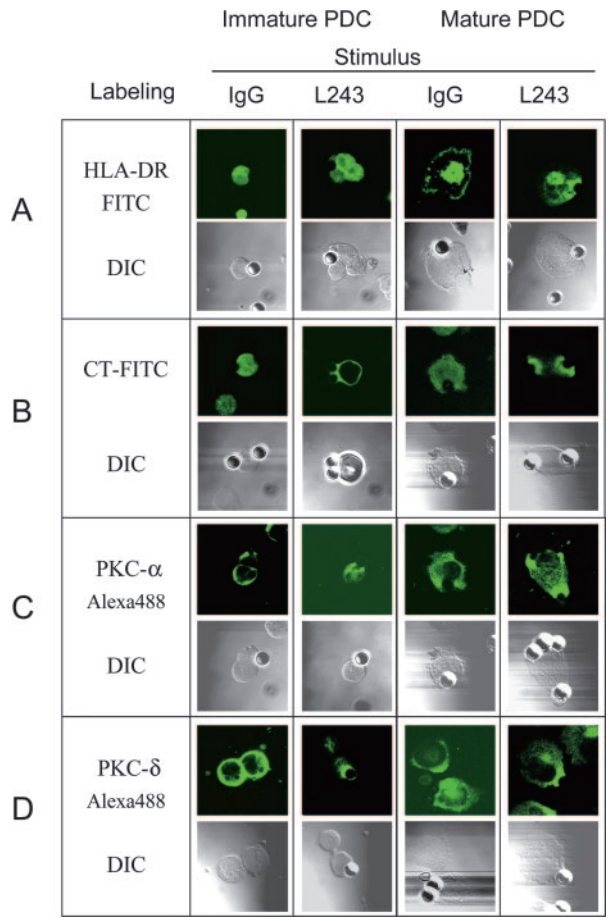

Fig. 2. Recruitment of HLA-DR, GM1, and PKC isoforms to the site of HLA-DR engagement in PDC, which were stimulated with HLA-DR mAb-coated or isotype-control, Ig-coated beads at a 1:2 ratio for 15 min. The stimulus is indicated at the top of each column. Freshly isolated cells were compared with CD40-matured PDC (i.e., cultured in the presence of IL-3 and CD40L for 48 h). Localization of the different molecules was detected by confocal microscopy after labeling using: HLA-DR (L243) mAb (A), CT to detect GM1 (B), anti-PKC-α (C), and anti-PKC-δ Ab (D). Each image of labeled cells is presented with the corresponding differential interference contrast (DIC) micrograph. (A) Diffuse HLA-DR expression was observed in freshly isolated PDC, whereas expression in mature PDC was clearly localized to the plasma membrane and to discrete intracellular compartment(s) (first and third microphotographs). Recruitment of HLA-DR molecules as a consequence of engagement with L243-coated beads was only observed in mature PDC (fourth microphotographs). (B) Lipid raft localization was detected by labeling GM1 with FITC-CT B subunit, and relocalization as a consequence of HLA-DR engagement was observed in mature and immature PDC. (C) PKC-α and (D) PKC-δ relocalization is observed in immature and mature PDC.

HLA-DR molecules to the specific site of HLA-DR engagement in the mature PDC. Representative images (n=3 donors) are shown in Figure 2A.

Localization of HLA class II molecules within GM1-containing lipid rafts has been described in MDC [16]. We therefore examined whether engagement of the HLA-DR molecules influenced the distribution of GM1 in the PDC. In contrast to HLA-DR, relocalization of the lipid raft marker GM1 to the site of HLA-DR engagement was observed in immature and mature PDC (Fig. 2B).

We next examined the PKC family of isoenzymes, which has been implicated in HLA-DR signaling in MDC [30, 32]. The

images in Figure 2 reveal that the PKC isoenzymes α and δ are recruited to the site of HLA-DR engagement in immature and mature PDC (Fig. 2, C and D).

Thus, these data reveal that despite the lack of detectable HLA-DR recruitment to the site of HLA-DR engagement in the immature PDC, signal transduction via HLA-DR takes place, leading to recruitment of lipid rafts and of PKC isoenzymes. In contrast, the engagement of HLA-DR molecules in the mature PDC leads to recruitment of HLA-DR, PKC isoenzymes, and lipid rafts to the site of HLA-DR engagement.

HLA-DR-induced cell death in PDC

Having observed that the HLA class II molecules expressed on the PDC were signal-transducing molecules, we next examined whether signaling via the HLA-DR molecules could transmit an apoptotic signal, as has been described in MDC. PDC apoptosis was analyzed after incubation with an anti-HLA-DR mAb or an IgG2a isotype control for 24 h using a plasma membrane readout of apoptosis (Annexin V staining). As immature PDC undergo apoptosis spontaneously in the absence of IL-3, immature and CD40-matured PDC were maintained in IL-3 containing medium throughout stimulation. Four conditions were tested, and cell death was determined in response to HLA-DR mAb or isotype-control Ig in each condition, as shown in **Figure 3**: Freshly isolated PDC were incubated with L243 or IgG2a for 24 h in IL-3-containing medium (PDC IL-3). Freshly isolated PDC were incubated with L243 or IgG2a for 24 h in IL-3 and CD40-containing medium (PDC IL-3+CD40). Freshly isolated PDC were maintained in IL-3-containing medium for 48 h followed by a further 24-h incubation with L243 or IgG2a (PDC mat IL-3). Freshly isolated PDC were fully matured in IL-3 and CD40L-containing medium for 48 h followed by a further 24 h incubation with L243 or IgG2a (PDC mat IL-3+CD40).

PDC, which had been cultivated in the presence of IL-3 alone or fully matured with CD40L, underwent a high level of apoptosis via HLA-DR (conditions 3 and 4 in Fig. 3). The mean level of HLA-DR-mediated apoptosis in IL-3-maintained PDC (n=4) and in CD40L-matured PDC (n=4) was 67.3% ± 13 and 65.5% ± 15.9, respectively, compared with 36.4% ± 13.7 and 33.6% ± 9.3 for freshly isolated PDC directly incubated with L243 in the presence of IL-3 or IL-3 and CD40L, respectively (n= 4). These percentages are representative of absolute numbers, as they were calculated by recording a high number of events (>2000) in the absence of gating and with a low FSC threshold, which allows detection of all dead cells (see Fig. 3A). Thus, freshly isolated, immature PDC were much less sensitive to HLA-DR-mediated apoptosis than partially matured PDC, which had been maintained in IL-3, or fully matured PDC, matured via CD40. Statistical analysis (Wilcoxon paired *t*-test) was carried out to compare L243 versus IgG2a-induced cell death in immature PDC, and no difference was detected (in the IL-3 or the IL-3 and CD40L-cultivated populations).

In contrast, when L243 versus Ig-induced apoptosis was compared in mature PDC (maintained in IL-3 alone or IL-3 and CD40L), a significant difference was observed ($P<0.001$ in either case).

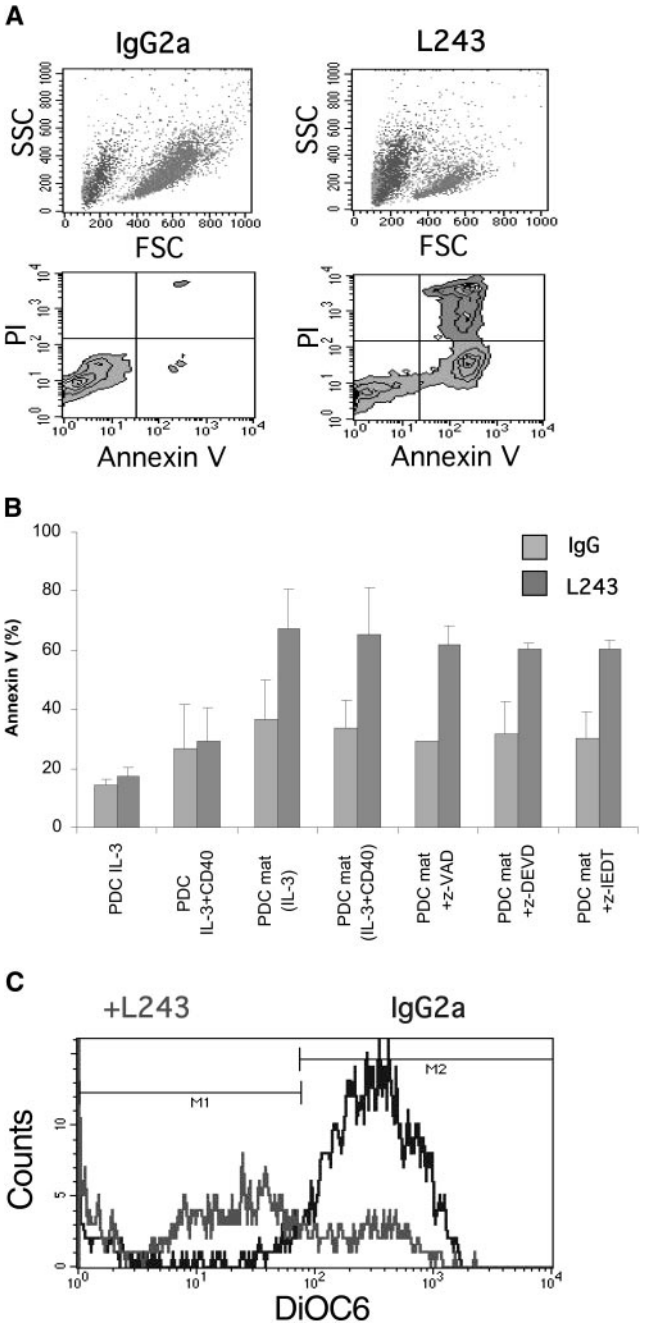

Fig. 3. HLA-DR-mediated apoptosis in PDC. (A) A forward- and side-scatter (FSC and SSC) plot of CD40-matured PDC incubated with Ig isotype control or HLA-DR mAb is shown. A typical Annexin-V-FITC and PI-staining profile is also shown. (B) Fresly isolated PDC directly stimulated via HLA-DR in the presence of IL-3 or IL-3 and CD40L did not undergo significant apoptosis compared with isotype control-stimulated PDC (Wilcoxon ranked *t*-test, n=4, conditions 1 and 2). PDC, maintained in IL-3 or matured via CD40 in IL3-containing medium, underwent significant apoptosis via HLA-DR ($P<0.001$, n=4, conditions 3 and 4) compared with isotype control-containing cultures. Addition of irreversible caspase inhibitors z-VAD, z-DEVD, and z-IETD failed to inhibit HLA-DR-mediated apoptosis of fully matured PDC (PDC mat; n=3). Means and SE are shown. (C) HLA-DR signaling initiates mitochondrial depolarization in mature PDC. A clear decrease in the $\Delta\psi m$ of mature PDC was detected after incubation with L243. The decreased level of $DiOC_6$ staining in the presence of L243 compared with the isotype control indicates mitochondrial transmembrane depolarization of the CD40-matured PDC. One of two independent experiments is shown.

We next determined whether HLA-DR-mediated apoptosis of CD40-matured PDC was PKC-dependent. HLA-DR-mediated death of mature PDC was not at all inhibited by the addition of the irreversible caspase inhibitors z-VAD, z-DEVD, and z-IETD (Fig. 3B). Under identical conditions, the same caspase inhibitors strongly inhibited CD95-mediated apoptosis of Jurkat T lymphocytes (data not shown).

As PKC has been implicated in HLA-DR-mediated apoptosis of MDC, CD40L-matured PDC were pretreated with the broad-spectrum PKC inhibitor Calphostin C (0–100 nM) before stimulation via HLA-DR. Unlike MDC, no difference whatsoever was observed between the apoptosis induced via HLA-DR at any of the concentrations tested for Calphostin C (data not shown, n=2).

HLA-DR engagement leads to mitochondrial depolarization in mature PDC

As neither caspase inhibition nor PKC inhibition altered HLA-DR-mediated apoptosis of mature PDC, we next tested the intrinsic pathway of apoptosis, indicated by mitochondrial transmembrane depolarization. Cells were incubated with L243 mAb or with an isotype-control IgG for 6 h prior to labeling with a marker of $\Delta\psi m$ $DiOC_6$. Figure 3C shows a marked decrease (71%) of the $\Delta\psi m$ of the CD40-matured PDC in response to signaling via HLA-DR, whereas PDC incubated with an isotype-control Ig control maintained a high level of fluorescence, indicating integrity of the transmembrane potential.

DISCUSSION

An increasingly documented characteristic of APC is their sensitivity to apoptosis after engagement of their MHC class II molecules [20, 21, 24, 29]. The physiological explanation for this response is likely to lie in the homeostasis of the immune response, as a large proportion of peptide-presenting APC undergo apoptosis after engagement with peptide-specific $CD4^+$ T lymphocytes [24, 25].

Although activated, mature PDC behave as APC, little is known of their response to antigen presentation via the MHC class II antigens. In this report, we reveal that in addition to the marked regulation of HLA-DR expression, PDC maturation also regulates HLA-DR-mediated signal transduction, indicated by an altered, functional outcome of HLA-DR engagement in terms of the acquired sensitivity of mature PDC to apoptosis. In this study, PDC were isolated by positive selection via BDCA-2. Signal transduction via BDCA has been previously reported to inhibit IFN secretion [33]. However, it is highly improbable that such signaling takes place under the conditions of the isolation procedure. Moreover, all cells were isolated by the same procedure; it is therefore unlikely that the BDCA-2 molecule intervenes in the different responses observed in mature versus immature cells.

In freshly isolated PDC, engagement of HLA-DR antigens did not lead to relocalization or clustering of the HLA-DR antigens to the site of engagement, whereas in mature PDC, clustering was clearly observed. However, despite the lack of relocalization of the HLA-DR molecules to the site of HLA-DR engagement in resting PDC, signal transduction was initiated from this site, as demonstrated by the recruitment of PKC isoenzymes. Therefore, signal transduction, indicated by PKC activation, does not rely on prior recruitment of the HLA-DR molecules to the site of engagement in immature PDC, and such signaling did not lead to apoptosis. In the mature PDC, recruitment of HLA-DR and PKC isoenzymes were all observed at the site of HLA-DR engagement. We have previously revealed that GM1-containing lipid rafts play a key role in HLA-DR-mediated signaling [34]. The recruitment of GM1 to the site of HLA-DR engagement in immature and mature PDC provides further evidence for signaling in immature PDC despite the lack of HLA-DR recruitment. The low numbers of PDC available did not allow us to study the role of PKC signaling, but downstream events following MHC class II-mediated signaling in nonterminally differentiated APC include cytokine production [35] and maturation [36].

Engagement of HLA-DR molecules by L243-coated beads provides a model for the MHC class II-TCR interaction, contributing to immune synapse formation. Our previous studies have implicated the actin cytoskeleton of the APC, PKC signaling in the APC, as well as APC lipid raft microdomains in the formation of the immunological synapse and optimal T lymphocyte activation [37]. It is interesting that the state of maturation of the T lymphocyte or the APC can alter the constituents and/or duration of the immune synapse [38, 39]. Modification of the immune synapse could provide at least one way of regulating downstream events such as polarization of effector cell function [40].

Extension of the types of MHC class II-expressing cells sensitive to HLA-DR-mediated apoptosis to include PDC provides a further argument for considering HLA-DR-mediated apoptosis as a key mechanism of regulating the survival of the APC, regardless of the nature of the APC. In common with MDC, activation and maturation of PDC are required for them to behave as APC, and the apoptosis induced as a consequence of HLA-DR engagement could therefore take place following peptide presentation, as has been demonstrated for MDC and for B lymphocytes [24, 25]. In common with MDC and B lymphoproliferations, the HLA-DR mAb-mediated apoptosis in PDC was not inhibited by blocking caspase activation. Moreover and in contrast to MDC, the broad-spectrum PKC inhibitor Calphostin C did not inhibit HLA-DR-mediated apoptosis in PDC. The intrinsic pathway of apoptosis could therefore be the major pathway in HLA-DR-mediated apoptosis of PDC.

Finally, as leukemic PDC behave similarly to mature PDC from healthy donors, humanized HLA-DR mAb could provide useful therapeutic agents for the treatment of the relatively rare PDC leukemias. Similar data concerning the more frequent B lymphocyte proliferations have been published, and such humanized ligands for the HLA class II molecules are under study as potential therapeutic tools [27]. The present study confirms the selectivity of the HLA class II Ab for inducing apoptosis of activated/mature cells. The resistance of $HLA\text{-}DR^+/CD34^+$ cells to HLA-DR-driven apoptosis has been documented [20, 21]. In any case, the continuation of haematopoiesis after in vivo treatment of B lymphoproliferations with HLA-DR mAb provides an argument supporting the develop-

ment of humanized HLA-DR ligands for the treatment of HLA class II molecule-expressing lymphoproliferations [27]. Similarly, in the course of B cell development, $CD34^+/CD19^+$ immature B cells and mature, normal $HLA\text{-}DR^-$ plasma cells will not be targeted by such treatments. Our in vitro data argue that immature PDC will also be resistant to HLA-DR-mediated apoptosis and support the development of HLA-DR ligands as therapeutic tools.

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