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Membrane Transfer from Tumor Cells Overcomes Deficient Phagocytic Ability of Plasmacytoid Dendritic Cells for the Acquisition and Presentation of Tumor Antigens

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The potential contribution of plasmacytoid dendritic cells (pDCs) in the presentation of tumor cell Ags remains unclear, and some controversies exist with regard to the ability of pDCs to phagocytose cell-derived particulate Ags and cross-present them to MHC class I–restricted T lymphocytes. In this study, we show that human pDCs, although inefficient in the internalization of cell membrane fragments by phagocytosis, can efficiently acquire membrane patches and associated molecules from cancer cells of different histotypes. The transfer of membrane patches to pDCs occurred in a very short time and required cell-to-cell contact. Membrane transfer also included intact HLA complexes, and the acquired Ags could be efficiently recognized on pDCs by tumor-specific CD8+ T cells. Remarkably, pDCs isolated from human colon cancer tissues displayed a strong surface expression of epithelial cell adhesion molecule, indicating that the exchange of exogenous Ags between pDCs and tumor cells also can occur in vivo. These data demonstrate that pDCs are well suited to acquire membrane patches from contiguous tumor cells by a cell-to-cell contact–dependent mechanism that closely resembles "trogocytosis." This phenomenon may allow pDCs to proficiently present tumor cell–derived Ags, despite limited properties of endophagocytosis. *The Journal of Immunology*, 2014, 192: 824–832.

Optimal activation of tumor-specific, cytotoxic CD8+ T cells classically requires coactivation of CD4+ Th lymphocytes and interaction with T cell costimulatory molecules. Because tumor cells usually lack both T cell costimulatory molecules and MHC II molecules, which are necessary for CD4+ T cell activation, the induction of antitumor T cell–based immunity classically requires professional APCs, such as dendritic cells (DCs) (1). Nevertheless, Ag-specific CD8+ T cells also can expand in vivo when exogenous tumor Ags are presented by DCs in the context of MHC class I molecules upon acquisition of dead or dying tumor cells, a phenomenon known as cross-priming or cross-presentation (2). More recently, a different mechanism involving direct membrane transfer of MHC–peptide complexes was hypothesized for tumor Ag presentation by DCs (3). Exchange of membrane molecules/Ags between immune cells has been observed for a long time (4, 5), but the mechanisms and functional consequences of these transfers have not been completely elucidated. Nevertheless, several studies (3, 6–9) reported that transfer of peptide–MHC complexes on DCs is a potential additional mechanism for activating T cells and referred to the process as "DC cross-dressing," a term originally coined by Yewdell and Haeryfar (10). Although cross-dressing has been clearly shown in conventional myeloid DCs (mDCs), this phenomenon has never been reported in plasmacytoid DCs (pDCs).

pDCs are a subset of DCs known to be endowed with the unique ability to produce large amounts of type I IFN in response to pathogen recognition (11). However, unlike mDC subsets, several features of the Ag-presenting potential of pDCs are still controversial, particularly in humans (12, 13). A point of contention relates to their ability to endophagocytose particulate Ags, such as artificial particles, cells, and bacteria (13). Therefore, the ability of pDCs to present or cross-present particulate Ags derived from tumor cells also remains to be clarified. In general, pDCs do not appear to be as endophagocytic as are mDCs (14–17), although other studies (18–20) came to the opposite conclusion. These discrepancies might be due to differences in the material used to assess pDC phagocytosis, as well as to divergences between the experimental animal models and the human system. It was proposed that uptake of exogenous Ags by pDCs, described in several studies, might occur primarily by micropinocytosis or surface receptor–mediated endocytosis (13). In contrast, pDCs might be able to endophagocytose in the presence of Ab-opsonized targets or if they receive signals through specific receptors (e.g., in the presence of microorganisms) (13, 21, 22).

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Abbreviations used in this article: BDCA2, blood dendritic cell Ag 2; B-EBV LCL, EBV-transformed B lymphoblastoid cell line; DC, dendritic cell; EpCAM, epithelial cell adhesion molecule; LCL, lymphoblastoid cell line; mDC, myeloid dendritic cell; ODN-A, oligodeoxynucleotide-A; pDC, plasmacytoid dendritic cell.

In this study, we aimed to understand to what extent human pDCs might be able to acquire exogenous materials from malignant cells. We found that, although human pDCs displayed limited competence in the internalization of tumor cell membrane fragments by phagocytosis, they were particularly suited to acquire tumor cell membrane patches and associated molecules, including HLA molecules, by a fast, cell-to-cell contact–dependent mechanism.

Materials and Methods
Ethics statement

This study was approved by the Ethical Committee of the Istituto Nazionale per la Ricerca sul Cancro (for cancer patient blood and tissues). All patients gave written informed consent according to the Declaration of Helsinki.

mAbs and flow cytometry

The following mAbs were used in the study: allophycocyanin- or FITC-conjugated anti- blood dendritic cell Ag 2 (BDCA2; clone AC144), PC5-conjugated anti-ILT3 (clone ZM3.8), allophycocyanin-H7–conjugated anti-CD45, allophycocyanin-conjugated anti-epithelial cell adhesion molecule (EpCAM; Miltenyi Biotec, Bergisch Gladbach, Germany), PE-conjugated anti-CD19, FITC-conjugated anti-CD3, PerCP-conjugated anti-CD8, PE-conjugated anti–IFN-γ, allophycocyanin-conjugated anti-CD11c, FITC-conjugated anti-CD14, and FITC-conjugated anti-CD56 (BD Bioscience, San Jose, CA). Anti–HLA-A1, anti-ICAM1, and anti-LFA1 were kindly provided by Dr. Alessandro Moretta (University of Genoa). For analysis of pDCs in tumors, cells were labeled with anti-CD45 and BDCA2 mAbs, and analysis was performed on CD45+ and BDCA2+ cells. For indirect immunofluorescence staining, nonspecific binding sites were saturated with human γ-globulin and then the relevant mAb was added and incubated for 30 min at 4°C. After washing, PE-conjugated isotype-specific goat anti-mouse Abs (Southern Biotechnology, Birmingham, AL) were added and incubated for 30 min at 4°C. Cells were then washed and analyzed by flow cytometry. Negative controls included directly labeled and unlabeled isotype-matched irrelevant mAbs. IFN-γ expression analysis was performed on CD3/CD8 double-positive cells by PE-conjugated reagents (BD Biosciences-Pharmingen) after cells were fixed in 2% paraformaldehyde and permeabilized with 0.1% saponin. Analyses were carried out using a flow cytometer (FACSCanto II; Becton Dickinson, Mountain View, CA). Data analysis was performed using FlowJo software 7.5.3 (TreeStar).

DC isolation and culture

Peripheral blood pDCs and mDCs were sorted by flow cytometry (FACSAria II; Becton Dickinson, Mountain View, CA) as BDCA2+ (or CD14− BDCA4+) cells and as lineage-negative (CD3, CD19, CD56, CD14) CD11c+ ILT3+ cells, respectively. Purity was ≥97%. In some instances, pDCs were also purified by negative selection using a pDC isolation kit and MACS LD columns (Miltenyi Biotec). Purified pDCs were cultured in RPMI 1640 (Euroclone, Milan, Italy), supplemented with 10% FBS (Euroclone), penicillin (100 U/ml), streptomycin (100 mg/ml), L-glutamine (2 mM), and IL-3 (20 ng/ml; all from R&D Systems, Minneapolis, MN). When indicated, 5 μg/ml CpG oligodeoxynucleotide-A (ODN-A) (TIB Molbiol, Genoa, Italy) was added to pDC cultures. Freshly purified pDCs or mDCs were cocultured with different tumor cell lines or autologous B cells and labeled with reagent dyes, according to the manufacturer's instructions, or with *Lactobacillus reuteri* (50:1 pHrodo-labeled bacteria/DC). Alternatively, DCs were cultured with freeze-thawed tumor cell fragments. Briefly, PKH-26–labeled lymphoblastoid cell line (LCL) tumor cells (5 × 10^6) were lysed by rapid freezing (-80°C) and thawing in a 37°C bath three times. The resulting suspension, containing only cell fragments, was centrifuged for 10 min in a microfuge at 7000 rpm. Pellets were used as a source of cell fragments. DCs were cultured with targets at an E:T (or tumor fragment equivalent) ratio of 1:1, for the indicated times, at 37°C in 5% CO2 at a concentration of 0.5 × 10^6 in a polystyrene tube (total volume 200 μl).

In selected experiments, pDCs were cocultured with tumor cells in the presence of blocking mAbs specific for ICAM-1, LFA-1, ILT2, total HLA class I (A6132) (kindly provided by Dr. Alessandro Moretta), and BDCA2 (AC144; Miltenyi Biotec). All relevant mAbs and related isotype-matched control Abs were added to the cultures at a concentration of 10 μg/ml.

To assess IFN-α production, after coculture with tumor cells, pDCs were stimulated with CpG ODN-A (5 μg/ml) for 12 h, in the presence of monensin (2 μM) and brefeldin (10 μg/ml) for the last 6 h, and IFN-α was detected by intracellular staining using an anti–IFN-α2b mAb (clone 7N4-1; Becton-Dickinson).

Cell lines

EBV-transformed B LCLs (B-EBV LCLs) were obtained from healthy donors, as previously described (23). A primary melanoma cell line was originated in our laboratory from a metastatic lesion of an HLA-A1+ patient that was obtained in accordance with consent procedures (n. OMA09.001) approved by the Internal Ethics Board of the Italian National Cancer Institute, Genoa. Tissue specimens were processed for the establishment of the cell line, as previously described (24). Human melanoma cell line FO-1 was kindly provided by S. Ferrone (Massachusetts General Hospital, Boston, MA), whereas the human colon carcinoma cell line Caco2, human leukemic monocyte lymphoma cell line U937, breast carcinoma cell line MCF-7, human leukemia cell line K562, and MHC class I–deficient B-EBV LCL 721-221 were purchased from the American Type Culture Collection and were used within 6 mo of resuscitation of original cultures.

Cell labeling with fluorescent dyes

Cells were labeled with PKH-26 (Sigma-Aldrich, St. Louis, MO), per the manufacturer's instructions. For labeling with pHrodo reagent dye, cells were washed twice in PBS and resuspended in PBS at 10^6 cells/ml. pHrodo-SE (stock solution in DMSO) was added to cell suspension at a final concentration of 2 μg/ml. After incubation for 30 min at room temperature, cells were washed twice with PBS and resuspended in complete RPMI 1640 medium. *L. reuteri* 5289 bacteria were labeled with amine-reactive pHrodo-SE (Sigma-Aldrich), per the manufacturer's instructions.

RT-PCR analysis

LCL or Caco2 cell lines and pDCs were sorted after 6 h of coculture. Total RNA was extracted using an RNeasy Micro Kit (QIAGEN, Hilden, Germany). cDNA was generated using a Transcriptor First Strand cDNA Synthesis Kit (Roche). Reverse transcription was performed at 25°C for 10 min and at 50°C for 60 min using total RNA extracted and random primers. PCR amplifications were performed for 30 cycles (30 s at 95°C, 30 s at 58°C, 30 s at 72°C) using Platinum TAQ (Invitrogen). The following primers were used: CD19: 5′-GTCTTATGAGAACGAGGATG-3′ (forward) and 5′-AGGAATACAAAGGGGACTGG-3′ (reverse), EpCAM: 5′-CGCAGCT-CAGGAAGAATGTG-3′ (forward) and 5′-ACGCGTTGTGATCTCCTTCT-3′ (reverse), and β-actin: 5′−ACTCCATCATGAAGTGTGACG-3′ (forward) and 5′−CATACTCCTGCTTGCTGATCC-3′ (reverse). β-actin amplification was performed using identical PCR conditions and served as an internal control. PCR products (249-bp fragment for β-actin, 488-bp fragment for CD19, and 456-bp fragment for EpCAM) were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

HLA typing

To perform HLA genomic typing, DNA was extracted using the QIAmp DNA blood mini kit (QIAGEN), according to the manufacturer's instruction. To identify HLA alleles, a PCR-SSO reverse assay was performed using HLA Kits (Innogenetics, Gent, Belgium). Data analysis was performed using LIPA Interpretation Software (Innogenetics).

Tissue processing

Surgically resected colon cancer samples were processed using a gentle-MACS Dissociator (Miltenyi Biotec), per the manufacturer's instructions. Briefly, surgical resections were processed in complete medium supplemented with Collagenase IV (1 mg/ml; Sigma-Aldrich) and DNase I (0.1 mg/ml; Worthington), using a gentleMACS dissociation program for human tumor tissues, and the cells were washed and stained for CD45, BDCA2, and EpCAM.

Confocal microscopy

For confocal microscopy analysis, sorted pDCs were cocultured for 6 h with a PKH-26–labeled LCL stained with anti-BDCA2. Then, cells were washed in PBS, placed onto poly-L-lysine–coated slides, and fixed. Fluorescent images were acquired on an inverted confocal microscope (LSM 510 DUO with 63×/1.40 oil-immersion objective; Carl Zeiss). Multiple color images were acquired by scanning in sequential mode to avoid cross-excitation. Samples were excited at 488 and 546 nm. Images were analyzed with Carl Zeiss software Zen 2011.

Multispectral-imaging flow cytometry

pDC acquisition of cell membrane patches was analyzed by ImageStream^x (Amnis, Seattle, WA), a multispectral flow cytometer combining standard microscopy with flow cytometry. It can acquire up to 100 cells/s, with simultaneously acquisition of six images of each cell, including bright field,

scatter, and multiple fluorescent images. We used the integrated software INSPIRE to run the ImageStream^x. For each experiment, cells (pDCs, LCL, and mDCs) were stained with respective markers or PHK-26 and suspended in 50 μl buffer (cold PBS with 1% FCS and 0.05% sodium azide) in 0.6-ml microcentrifuge tubes. Before running the samples, the ImageStream^x was calibrated using SpeedBeads. Samples were acquired in the following order: unlabeled, single-color fluorescence controls, and finally, the experimental samples. Samples were always left on ice. At least 10,000 cells/experimental sample and 2,000 cells/single-color control were acquired for each sample. After each sample was injected into the flow cell, the cells were allowed to form a single core stream before acquisition. Images were analyzed using IDEAS image-analysis software (Amnis).

Immunohistochemistry

For EpCAM/BDCA2 (CD303) double staining, paraffin-embedded tissue sections of colon carcinoma were deparaffinized, treated with H_2O_2/3% for 5 min to inhibit endogenous peroxidase, and washed in H_2O. They were incubated for 30 min with the first primary Ab (mouse monoclonal, clone 124B3.13; Dendritics, Lyon, France), followed by detection with the Bond Polymer Refine Detection Kit (Leica Biosystems), according to the manufacturer's protocol, and subsequently were incubated for 30 min with the second primary Ab (rabbit monoclonal, clone EPR677; Abcam, Cambridge, U.K.), followed by detection with the Bond Polymer Refine Red Detection Kit (Leica Biosystems), according to the manufacturer's protocol.

Assessment of tumor-associated Ag recognition on cross-dressed pDCs

To evaluate whether T cells were able to recognize the transferred HLA/Ag complexes on pDCs, we established EBV-specific CD8$^+$ T cell clones, as previously described (25), and used B-EBV LCL as a source of Ags from tumor cells.

Two HLA class I–restricted T cell clones specific for EBV proteins were used: one restricted by HLA-A2 and recognizing LMP2 protein (CLGGLLTMV epitope, HLA-A2/LMP2$_{426-434}$) and the other restricted by HLA-B8 and recognizing EBNA3A (FLRGRAYGL epitope, HLA-B8/EBNA3A$_{325-333}$). To obtain B-EBV LCL suitable as a source of Ag for T cell clone recognition, B lymphocytes from normal donors expressing either HLA-B8 or HLA-A2 were immortalized with B-EBV LCL, as previously described (23).

Then, pDCs were isolated from three donors not expressing HLA-B8 or HLA-A2. In each experiment, pDCs were cocultured with either HLA-B8$^+$/A2$^+$ LCLs or with LCL not expressing the relevant HLA class I molecules. After 6 h of pDC/LCL coculture, pDCs were highly purified by flow cytometry (>98% in all experiments) and used as stimulators of the EBV-specific T cell clones at an stimulator/effector ratio ranging from 1:10 to 1:1. To exclude possible LCL contamination in sorted pDCs, an aliquot of pDCs was always cultured in complete medium and the putative growth of LCL was monitored for an additional 3 wk.

IFN-γ production by CD8$^+$ T cell clones was analyzed upon stimulation with EBV-specific relevant peptide, irrelevant peptide, HLA-B8$^+$/A2$^+$ LCL (as positive control), HLA-B8$^-$/A2$^-$ LCL (as negative control), sorted pDCs after coincubation with HLA-B8$^+$/A2$^+$ LCL, and sorted pDCs after coincubation with HLA-B8$^-$/A2$^-$ LCL. IFN-γ production by T cell clones was evaluated by both intracytoplasmic staining, as previously described (26), and commercial ELISA (R&D Systems) 6 and 24 h after stimulation, respectively.

Results

pDCs acquire tumor cell membrane patches independently of phagocytosis

It has been a matter of controversy whether human pDCs capture "cell-derived" antigenic material like conventional mDCs do. Therefore, we investigated the capacity of pDCs to capture and internalize cell components.

For this, cancer cell lines derived from different tissues were loaded with the fluorescent dye PKH-26, which stably incorporates into lipid regions of the cell membrane. pDCs acquired PKH-26 fluorescence as early as 30 min after coculture with PKH-26–loaded cancer cells (pDC/cancer cell ratio, 1:1), thus indicating that pDCs can acquire cancer cell membrane components in a short interval of time (Fig. 1A). Interestingly, when normal B cells and autologous B-EBV LCLs were compared, only the neoplastic

counterpart could be acquired by pDCs (Fig. 1B). However, further analyses by confocal microscopy (Fig. 1C), as well as by multispectral imaging flow cytometry (Fig. 1E, *upper two panels*), revealed that PKH-26 fluorescence acquired by pDCs appeared to be restricted to the cell membrane, and not within the cytoplasm, and mainly were in the form of cell membrane patches. Multispectral-imaging flow cytometry was performed by ImageStream^x (Amnis), a bench-top instrument designed for the acquisition of six channels of cellular imagery. It combines the per-cell information content provided by standard microscopy with the statistical significance afforded by the large sample sizes common to standard flow cytometry. With this system, fluorescence intensity measurements are acquired in the same way as with a conventional flow cytometer; however, it takes advantage of the system's imaging abilities to locate and quantify the distribution of signals on or within cells.

To achieve further confirmation on whether pDCs could internalize tumor cell membrane–derived material, we repeated the experiments by labeling tumor cells with pHrodo, a cell linker dye that exhibits a minimal fluorescence at neutral pH and a consistent fluorescence only when internalized in acidic phagocytic endosome compartments. Fluorescence was only detected on pDCs following coculture of tumor cells labeled with PKH-26, but not with pHrodo, suggesting that the acquired material was not internalized by phagocytosis (Fig. 1D, *left panels*). Further supporting this hypothesis, tumor cell membrane components were acquired by pDCs only when they were incubated with viable tumor cells and not with cell fragments obtained by freezing/thawing cycles. On the contrary, as expected, tumor cell fragments were acquired efficiently by autologous peripheral blood mDCs (Fig. 1D, *right panels*, Fig. 1E, *lower two panels*). These results indicated that pDCs could acquire foreign cell membrane patches but without internalizing them by phagocytosis. The deficit in phagocytosis was also confirmed by the inability of pDCs to phagocytose bacteria, in sharp contrast to autologous peripheral blood mDCs (Fig. 1F).

The acquisition of cell membrane patches by pDCs requires IL-3 and cell-to-cell contact

The acquisition of cell membrane components by pDCs represents an active and dynamic process that was inhibited at low temperature (Fig. 2A). In addition, the presence of IL-3, which is necessary for the viability of pDCs in vitro (15), was required for the acquisition of cell membrane patches from tumor cells (Fig. 2B). To determine whether the ability to acquire tumor membranes was affected by the activation status, pDCs were cocultured for 6 h with the TLR-9 agonist CpG ODN-A in the presence of PKH-26–loaded LCLs. Overnight culture under the same conditions resulted in a slight, but significant, increase in tumor cell membrane acquisition (Fig. 2B).

Production of type I IFN upon TLR7/9 stimulation is a peculiar functional feature of pDCs. Thus, we investigated whether the acquisition of tumor cell membrane patches could interfere with IFN-α production. As shown in Fig. 2C, pDCs that had or had not acquired tumor cell membrane patches eventually were able to secrete IFN-α, upon CpG stimulation.

Tumor cell membrane transfer to pDCs appeared to be dependent on cell-to-cell contact, because it was completely abrogated in a Transwell device system (Fig. 2A). In support of this assumption, we found that blocking relevant adhesion molecules by specific mAbs—ICAM-1 and LFA-1—strongly affected the transfer to pDCs (Fig. 2D). In agreement with the increased capability of activated pDCs to acquire foreign membrane components, the two adhesion molecules were upregulated upon activation with TLR-9 ligand (Fig. 2E).
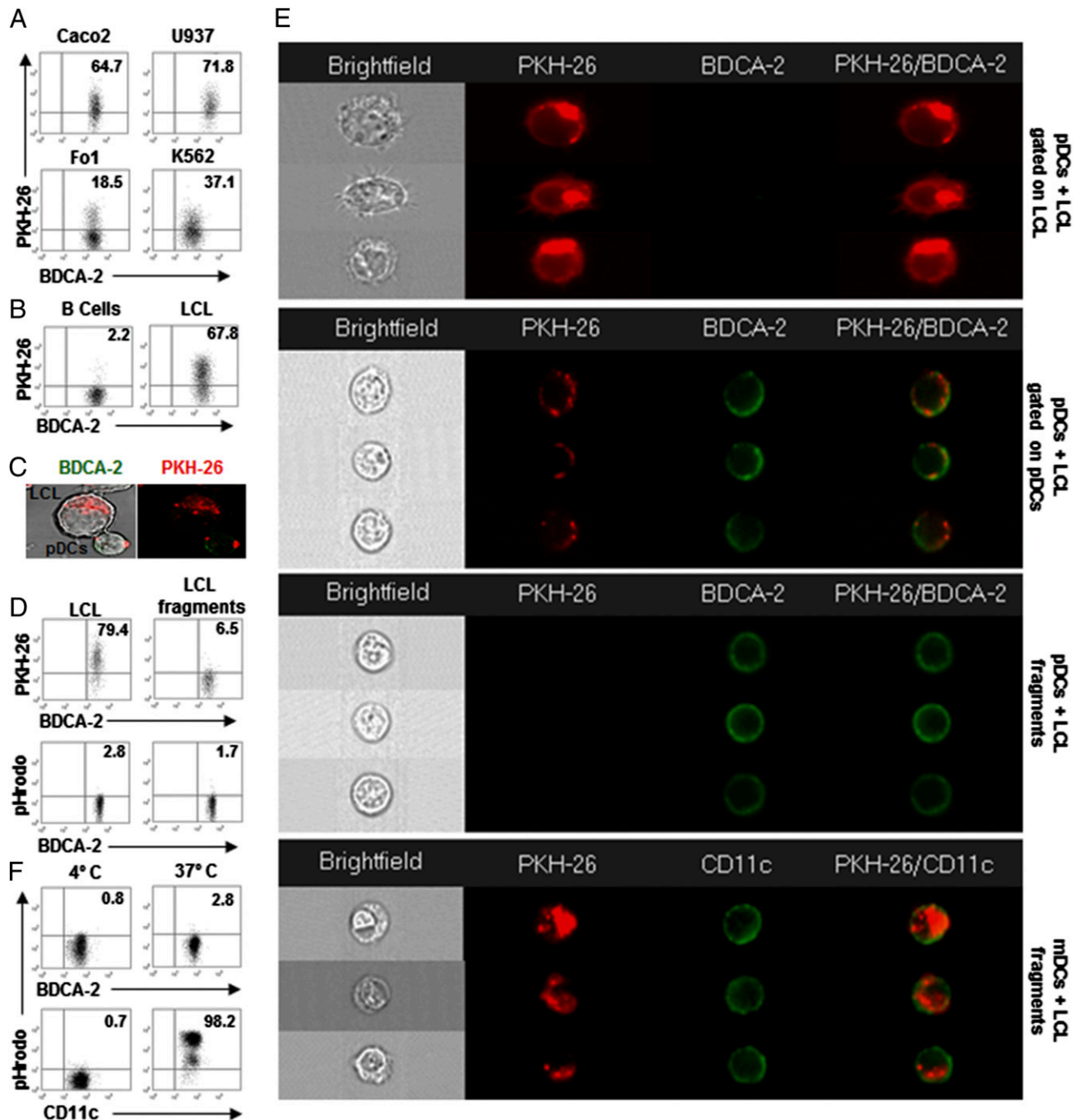
FIGURE 1. pDCs efficiently acquire membrane patches from tumor cells independently of phagocytosis. (**A** and **B**) pDCs were cocultured with PKH-26–labeled tumor cell lines of different histotypes, or normal B cells, and PKH-26 fluorescence was analyzed on pDCs gated according to physical parameters and BDCA2 expression. The values reported in the *top right corners* indicate the percentage of PKH-26$^+$ pDCs after 6 h of coculture. The experiment shown is representative of five independent experiments performed using different donors. (**C**) Confocal microscopy: pDCs were cocultured for 6 h with PKH-26–labeled LCLs, stained with anti-BDCA2, laid onto poly-L-lysine–coated slides, fixed, and analyzed (original magnification ×63, 1.4 oil-immersion objective). Green fluorescence corresponds to BDCA2; red fluorescence corresponds to PKH-26. (**D**) LCLs were labeled with PKH-26 (*upper panels*) or pHrodo (*lower panels*) reagent dyes, cocultured for 6 h with purified pDCs, and analyzed by flow cytometry (*left panels*). Alternatively, PKH-26– or pHrodo-labeled LCLs underwent repeated freeze-thaw cycles, and cell fragments were added to purified pDCs and incubated for 6 h (*right panels*). One experiment representative of three is shown. (**E**) ImageStream multispectral imaging flow cytometry: purified pDCs or circulating blood mDCs were cocultured with PKH-26–labeled LCLs (*upper two panels*) or with PKH-26–labeled LCLs that had undergone freezing and thawing cycles (LCL fragments) (*lower two panels*). Subsequently, cells were stained with anti-BDCA2 mAb and analyzed as described in *Materials and Methods* (original magnification ×40). (**F**) Purified pDCs (*upper panels*) or circulating blood mDCs (*lower panels*) were cultured for 6 h with pHrodo-labeled *Lactobacillus reuteri*, and acquisition of fluorescence was analyzed by flow cytometry. Cells incubated for 6 h with labeled *Lactobacillus reuteri* at 4˚C were also analyzed and used as negative control. These experiments are representative of three independent experiments performed with different donors.

Because pDCs express on their surface inhibitory molecules that might affect their functions, we investigated whether the inhibitory molecules BDCA2 and ILT-2 might impair this ability of acquiring membrane patches. BDCA2 binds to asialo-galactosyl-oligosaccharides that could be overexpressed by some tumor cells and this binding can suppress IFN-α production by pDCs (27).
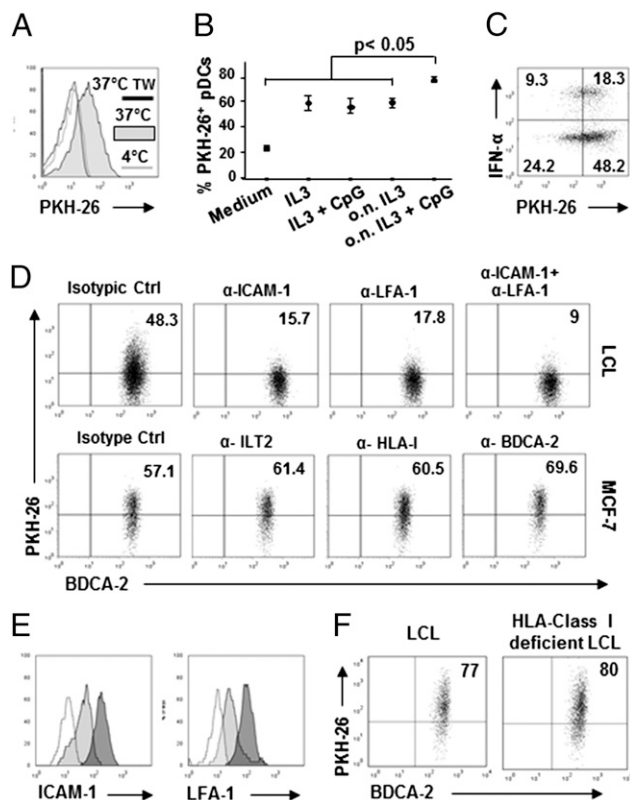
FIGURE 2. pDCs acquire membrane patches by a cell-to-cell contact–dependent mechanism. (**A**) pDCs were cocultured with PKH-26–labeled LCLs either separated (open graph, black line, 37°C TW) or not (filled graph, 37°C) by a Transwell device. pDCs cultured for 6 h with PKH-26–labeled LCLs at 4C° were also analyzed (open graph, gray line, 4°C). One experiment representative of three is shown. (**B**) pDCs were cultured with PKH-26–labeled LCLs in complete medium, with or without IL-3 or IL-3 plus CpG ODN-A, and then analyzed by flow cytometry for PKH-26 fluorescence. Alternatively, pDCs were incubated overnight (o.n.) in the presence of IL-3 or IL-3 plus CpG ODN-A, and PKH-26–labeled LCLs were added to the culture for an additional 6 h. (**C**) pDCs were cultured in the presence of PKH-26–labeled LCLs and then stimulated by CpG ODN-A for 12 h, when IFN-α by pDCs was analyzed by intracytoplasmic staining. (**D**) pDCs were cultured for 6 h with p-KH-26–labeled LCLs (*upper panels*) or MCF-7 (*lower panels*) in the presence of mAbs specific for the indicated molecules or isotype-matched irrelevant mAbs and then analyzed by flow cytometry. Values reported in the *top right corners* indicate the percentage of PKH-26$^+$ pDCs. (**E**) Adhesion molecule expression was analyzed in nonactivated pDCs (light gray–filled graphs) and upon activation by CpG ODN-A (dark gray–filled graphs). Open graphs represent staining with isotype-matched control mAbs. (**F**) pDCs were cultured in the presence of LCL (*left panel*) or 721-221 cells, an LCL not expressing HLA class I molecules (*right panel*). Both cell lines were labeled were PKH-26.

Therefore, we performed pDC/tumor cell coculture using MCF-7 cell line, a breast carcinoma cell line reported to express high levels of asialo-galactosyl-oligosaccharides (27). pDCs were able to efficiently acquire MCF7 membrane patches and blocking BDCA2 by a specific mAb did not affect the acquisition (Fig. 2D). Similarly, blocking mAbs against ILT-2 has no effect on membrane patches acquisition. In addition, we also blocked ILT2 ligand (i.e., HLA class I molecules), by the addition of the anti-total HLA class I–blocking mAb A6132. Therefore, ILT2/HLA interactions do not seem to affect pDC acquisition of tumor cell membranes (Fig. 2D).

To further confirm this, we analyzed pDC acquisition of tumor cell membrane patches from a B-EBV LCL that does not express

HLA class I molecules—721.221 cell line—and compared it with other LCL-expressing HLA class I molecules. pDCs were able to acquire a similar amount of membrane patches for donor cells when cocultured with either LCLs expressing normal levels of HLA class I or when cocultured with HLA class I–deficient LCLs (Fig. 2F).

Membrane Ags and HLA molecules from tumor cells can be transferred intact to pDCs

Next, we investigated whether the transfer of tumor cell membrane patches might result in the presence of tumor-associated molecules on the surface of pDCs. For this, pDCs were cocultured with B cell–derived LCLs, as well as with the colon carcinoma Caco2 cell line, for short intervals of time. Then, the expression of the B cell marker CD19 and EpCAM were assessed on pDCs by flow cytometry. As shown in Fig. 3A, pDCs acquired a strong surface expression of CD19 and EpCAM when cocultured with LCL and Caco2 cell lines, respectively. As expected, pDCs cocultured with these tumor cell lines did not express mRNA for either CD19 or EpCAM (Fig. 3B).

To address whether the acquisition of tumor Ags observed in vitro might also occur in vivo, we analyzed the expression of the epithelial-specific Ag EpCAM on pDCs infiltrating colon cancer carcinomas. We found that, although pDCs were located primarily at the periphery of EpCAM$^+$ colonic adenocarcinoma areas, interspersed in the lamina propria, they can be observed in tight contact with EpCAM$^+$ cells (inset, Fig. 3C). Remarkably, pDCs infiltrating the tumors exhibited a relevant surface expression of EpCAM, which was never detectable in autologous blood-circulating pDCs (Fig. 3D).

The transfer of intact molecules from tumor cells might have obvious functional consequences (e.g., presentation of tumor-associated Ags). To investigate this issue, we first analyzed whether HLA complexes could also be transferred from tumor cells to pDCs. Because most tumors do not express HLA class II, we were primarily interested in investigating the transfer of HLA class I complexes. Thus, pDCs obtained from an HLA-A1$^-$ donor were cultured with B-EBV LCLs expressing HLA-A1. Following coculture, pDCs were analyzed by flow cytometry for HLA-A1 expression and were found to have acquired HLA-A1 molecules (Fig. 3E, *upper panels*). Similar results were confirmed using a primary melanoma cell line established in our laboratory from an HLA-A1$^+$ patient (Fig. 3E, *lower panels*). HLA-A1 molecules were already present on pDCs after 30 min of coculture, reaching a plateau of expression at ~20 h. When pDCs were sorted from HLA-A1$^+$ tumor cells after 6 h of coculture, the expression of HLA-A1 molecules remained on pDCs for at least an additional 48 h, although they decreased progressively (Fig. 3F).

Tumor-specific CD8$^+$ T cells recognize Ags on cross-dressed pDCs

The ability of pDCs to cross-present exogenous Ags for the activation of CD8$^+$ T cells is still a matter of debate (13, 28). We investigated whether transferred HLA class I molecules from tumor cells to pDCs might directly allow the presentation of tumor-associated Ags to CD8$^+$ T cells. For this, we established CD8$^+$ T cell clones specifically recognizing EBV-associated proteins in B-EBV LCLs. Two T cell clones were eventually used in the study: one restricted by HLA-A2 and recognizing LMP-2 protein (CLGGLLTMV epitope, HLA-A2/LMP2$_{426–434}$) and the other restricted by HLA-B8 and recognizing EBNA3A (FLRGRAYGL epitope, HLA-B8/EBNA3A$_{325–333}$). Then, pDCs were isolated from three donors not expressing HLA-B8 or HLA-A2. In each experiment, pDCs were cocultured with either HLA-B8$^+$/A2$^+$ LCLs or, as a control, with an LCL not expressing the relevant HLA class I molecules.
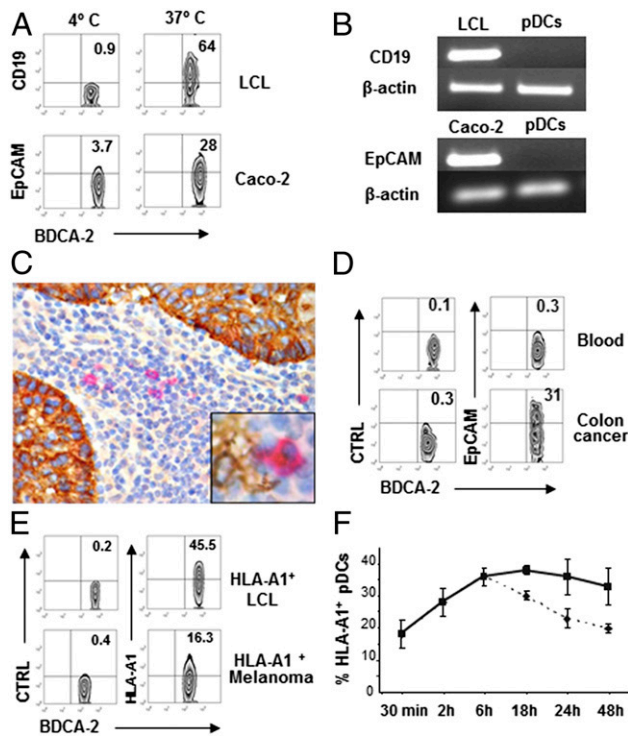
FIGURE 3. Acquisition of tumor cell membrane Ags by pDCs. (**A**) pDCs were cultured with B-EBV LCLs or Caco2 cell line. After extensive washing, cells were stained with anti-BDCA2 and either anti-CD19 or anti-EpCAM, respectively. Cells stained with isotype-matched mAbs were considered negative controls. The experiment shown is representative of five independent experiments performed using different donors. (**B**) pDCs that had been cocultured with B-EBV LCL or Caco2 were isolated by flow cytometry cell sorting and analyzed by RT-PCR for CD19 and EpCAM mRNA expression, respectively. B-EBV LCL and Caco2 cell lines were used as positive controls. (**C**) Immunohistochemical features of pDCs infiltrating colon adenocarcinoma. EpCAM⁺ (brown) colon cancer showing BDCA2⁺ pDCs (fuchsia) located primarily at the periphery, usually in the lamina propria adjacent to cancer (original magnification ×400). Close contact between EpCAM⁺ cancer cells and BDCA2⁺ pDCs also was detected (*inset*: original magnification ×1000). For EpCAM/BDCA2 double staining, deparaffinized tissue sections of colon carcinoma were incubated with anti-BDCA2 mAb (clone 124B3.13) followed by detection with the Bond Polymer Refine Detection Kit (Leica Biosystems). Subsequently, sections were incubated with anti-EpCAM mAb (clone EPR677), followed by detection with the Bond Polymer Refine Red Detection Kit (Leica Biosystems). (**D**) pDCs were isolated from either peripheral blood or colon cancer tissues and analyzed by flow cytometry for surface expression of EpCAM. The experiment shown is representative of three independent experiments performed with cancer tissues from different patients. (**E**) pDCs from an HLA-A1⁻ donor were sorted and cultured for 6 h with LCL derived from an HLA-A1⁺ donor (*upper panels*) or a primary melanoma cell line obtained from an HLA-A1⁺ patient (*lower panels*). pDCs were stained with anti-BDCA2 and anti–HLA-A1 mAbs or isotype-matched mAbs (CTRL) and analyzed by flow cytometry. The experiment shown is representative of five independent experiments performed using different pDC donors. (**F**) pDCs from an HLA-A1⁻ donor were cocultured with HLA-A1⁺ LCL, and acquisition of HLA-A1 on pDCs was measured by flow cytometry at 30 min and at 2 and 6 h. After 6 h, BDCA2⁺ pDCs were sorted from part of the pDC/LCL coculture, and HLA-A1 expression was further monitored by flow cytometry for up to 48 h in either sorted pDCs (dotted line) or pDCs maintained in culture with LCL (solid line). Data are mean and SD of an experiment performed in triplicate.

pDCs cocultured with B-EBV LCL from HLA-B8⁺/A2⁺, but not with LCL not expressing the relevant HLA class I molecules, induced IFN-γ production by the T cell clones (Fig. 4). To exclude

possible LCL minimal contaminations in sorted pDCs, an aliquot of sorted pDCs was cultured in complete medium for each experiment, and the putative growth of LCLs was monitored for three additional weeks.

As a whole, these results indicate that membrane transfer from tumor cells can allow pDCs to present tumor-derived HLA/Ag complexes to tumor-specific T cells.

Discussion

The transfer of MHC complexes to APCs has been termed "cross-dressing" (10), and it implies the generation of peptide–MHC complexes within the target cell and their subsequent transfer to APCs, which then present the intact, unprocessed complexes to T lymphocytes. In this study, we provided evidence for the transfer of intact membrane proteins from tumor cells to pDCs, including intact HLA complexes that can be recognized by T cells. The mechanism of transfer appeared to be dependent on cell-to-cell contact and closely resembled "trogocytosis," a neologism coined to indicate a well-documented mechanism of intercellular exchanges of intact membrane patches among cells (29). As a consequence, pDCs may become APCs for tumor-associated Ags. These findings have major implications for the evolution of specific antitumor immune response, also offering an additional explanation for the previously demonstrated capability of pDCs to present exogenous Ags to CD8⁺ T cells (12, 13, 18).

In general, pDCs do not appear to be as endocytic as mDCs, although this is still a matter of contention. There has been some controversy about whether pDCs are involved in the uptake of cell fragments or bacteria. Our study demonstrated that pDCs barely take up either bacteria or tumor cell fragments compared with mDCs. The situation might be different in the presence of microorganisms able to specifically trigger pDC endophagocytosis or in the presence of Ab/Ag immunocomplexes that can be endocytosed by pDCs in vivo (18, 19, 21, 22, 30). It was reported recently that pDCs exposed to gp100 protein–expressing BLM melanoma cell lysates were able to activate gp100-specific CD8⁺ T cells (31). However, internalization of cell membrane fragments was not shown, and the study did not clarify whether soluble gp100 protein might be present in cell lysates. Whatever the case, HLA/Ag transfer from tumor cells, described in this study, might represent an effective mechanism used by pDCs to present tumor Ags to specific CD8⁺ T cells.

We speculate that a similar mechanism of Ag transfer can occur in vivo and may account for the expression of epithelial cell–specific Ag that we observed on the plasma membrane of colon cancer–infiltrating pDCs. In addition to colon cancer, pDCs have been found in several other types of tumors: head and neck cancer, ovarian cancer, melanoma, and breast cancer (32–37). In tumors, the presence of pDCs correlated with an unfavorable prognosis, and it is thought to promote T cell tolerance rather than T cell activation (35, 37–40). pDCs were recently shown to be endowed with tolerogenic properties by inducing T cell anergy (19, 41–46). Although, as in the case of mDCs, the factors that determine whether the T cells primed by pDCs will differentiate into effector or regulatory T cells remain ill defined, the consensus is now that they can activate naive T cells, as well as memory T cells, if they undergo maturation (13, 47). Most likely, pDCs infiltrating tumors are not properly activated because of the lack of danger signals (33, 48); therefore, a tolerogenic role for tumor-infiltrating pDCs may be envisaged. In contrast, recent studies showed that in vivo TLR triggering with TLR ligands, such as CpG, induced pDC activation, resulting in antitumor immunity and partial tumor regression (49, 50). Furthermore, activated human pDCs pulsed with a tumor Ag can prime IFN-γ–secreting Ag-specific CTLs (34). We
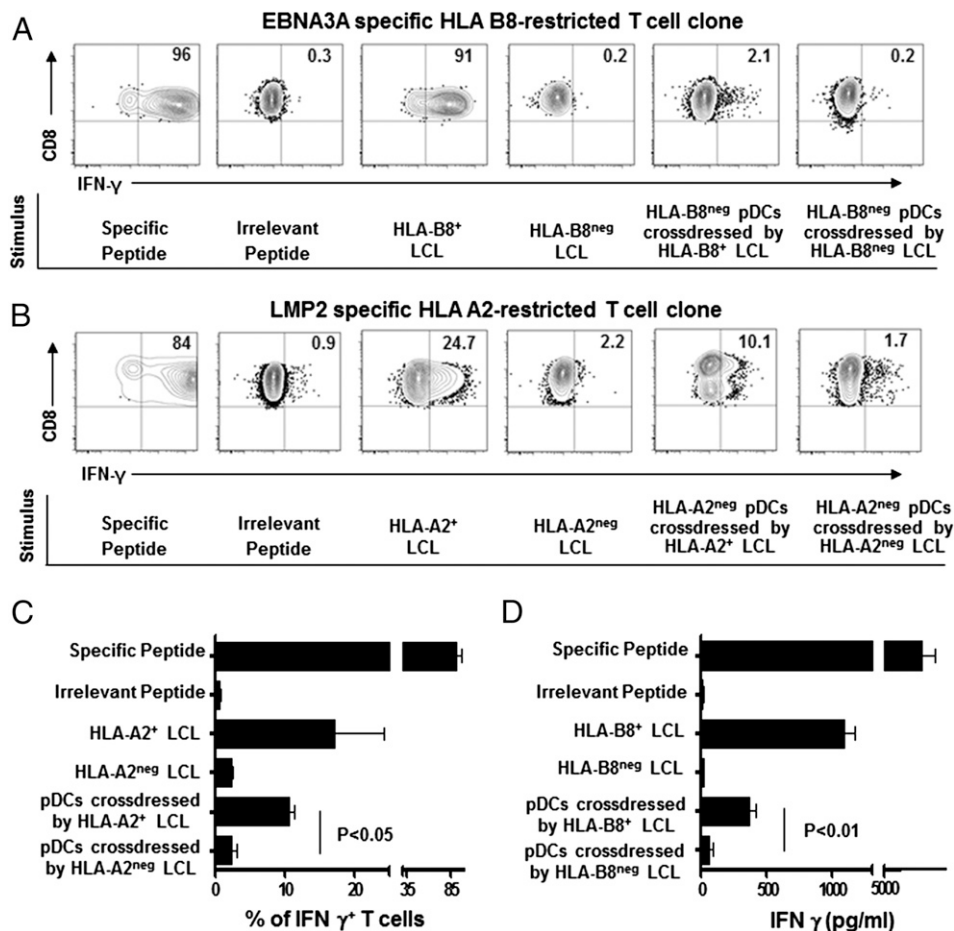
FIGURE 4. Recognition of tumor Ags on cross-dressed pDCs by HLA class I–restricted T cells. T cell clones specific for EBV Ags EBNA3A (**A**, **D**) and LMP2 (**B**, **C**) were stimulated by pDCs cross-dressed by B-EBV LCLs expressing or not expressing the HLA class I alleles restricting the T cell clones. Recognition of EBV-associated Ags on pDCs by T cells was identified by IFN-γ production by T cells either via intracytoplasmic staining (A–C) or by assessing IFN-γ release in T cell/pDC coculture supernatants by ELISA (D). As positive and negative controls, T cell clones were also stimulated by either specific or irrelevant peptides, as well as by LCLs used for pDC cross-dressing, either expressing or not expressing the HLA class I alleles restricting the T cell clones. Experiments in (A) and (B) are representative of three independent experiments of intracytoplasmic staining for IFN-γ. Data in (C) represent mean and SD of the percentage of T cells producing IFN-γ in three independent experiments with a T cell clone restricted by HLA-A2 and recognizing LMP-2 protein (CLGGLLTMV epitope, HLA-A2/LMP2$_{426-434}$), as representatively shown in (B). (D) IFN-γ produced by a T cell clone, restricted by HLA-B8 and recognizing EBNA-3 EBNA3A (FLRGRAYGL epitope HLA-B8/EBNA3A$_{325-333}$), was analyzed by ELISA in culture supernatant after 24 h. Data are mean and SD of three different independent experiments using pDCs by different donors not expressing the HLA-B8 allele.

showed in this study that pDCs, upon acquisition of functional Ag–MHC complexes from tumor cells by membrane uptake, can also present the acquired Ags to T cells. In addition, pDCs retained the acquired Ags on their cell surface 48 h after contact with tumor cells, thus potentially allowing an efficient presentation of the Ags in vivo. Interestingly, the acquisition of Ags from tumor cells did not require activation of pDCs, and this might predict a possible tolerogenic role for these cells in the context of tumor microenvironment. It is noteworthy that most human cancers do not express HLA class II molecules, thus mainly restricting pDCs cross-dressing of tumor cell Ags to HLA class I presentation. Therefore, the presentation of cross-dressed tumor Ags by pDCs might result in anergy of tumor-specific CTLs. In contrast, the stimulation of cross-dressed pDCs with a TLR ligands, such as CpG, would result in fully functional pDCs capable of stimulating antitumor responses via both production of IFN-α and direct induction of Ag-specific T cell responses. We demonstrated that pDCs that underwent cross-dressing with tumor Ags are not impaired with regard to their IFN-α production. These scenarios are even more intriguing considering the recent observation that pDCs can leave peripheral tissues and migrate via afferent lymph to

secondary lymphoid organs (51), although this remains to be demonstrated in humans. Nevertheless, pDCs can also play a relevant role in Ag presentation and/or immunomodulation directly at tumor sites, without necessarily reaching local lymph nodes for the presentation of cross-dressed Ags to T cells. In fact, several reports (41, 43, 44) indicated that pDCs can induce T cell tolerance, primarily through the induction of regulatory T cells. This may enable pDCs to promote regulatory T cell formation within the tumor site and to dampen the antitumor immune response of tumor-specific T cells (52).

In conclusion, the trogocytosis of tumor cell membranes may provide a unique pathway for the abundant presentation of tumor Ags by pDCs. The outcome of tumor Ag presentation by pDCs remains to be elucidated, because pDCs might have either tolerogenic or protective immune functions. However, considering the tolerogenic properties of pDCs at steady state (41, 45, 46) and the negative correlation of pDC infiltration and tumor prognosis (35, 37–39), tumor Ag cross-dressing of pDCs might represent a detrimental event. At the same time, strategies aiming to activate intratumoral pDCs (e.g., with CpG) might offer a tool for obtaining fully functional pDCs capable of stimulating antitumor responses

via both production of type I IFN and direct induction of tumor-specific T cell responses.

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

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