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Direct proteasome-independent cross-presentation of viral antigen by plasmacytoid dendritic cells on major histocompatibility complex class I

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Abstract

Although plasmacytoid dendritic cells (pDCs) respond to virus replication in a non-specific fashion by producing large amounts of type I interferon, a rapid, direct role of pDCs in activating antiviral lymphocytes is less clear. Here we showed that pDCs possess the capacity to rapidly initiate antigen-specific antiviral CD8⁺ T cell responses. Following virus exposure, pDCs efficiently and rapidly internalized exogenous viral antigens and then presented those antigens on major histocompatibility complex (MHC) class I to CD8⁺ T cells. Processing of exogenous antigen occurred within endocytic organelles and did not require transit of antigen to the cytosol. Intracellular stores of MHC class I partially colocalized with transferrin receptor and internalized transferrin in endosomes, suggesting that such recycling endosomes are sites of peptide loading onto MHC class I or peptide transit. These data demonstrate that pDCs utilize ready-made stores of MHC class I to rapidly present exogenous antigen to CD8⁺ T cells.

The high rate of viral replication represents a considerable challenge for the immune response. Human DCs can be classified into two major cell subsets, myeloid and plasmacytoid DCs, both of which are critical for the initiation of viral immune responses^{1,2}. The presentation of viral peptides to CD4⁺ and CD8⁺ T cells is mediated by major histocompatibility complex (MHC) class II molecules and class I, respectively. DC maturation leads to a variety of changes including, activation induced antigen processing and increased surface expression of MHC class I and class II^{3,4}. Exogenous antigen presentation on MHC class II molecules by myeloid DC (mDCs) is a rapid and coordinated process⁵. Pools of pre-synthesized MHC class II are stored in late endosomal and lysosomal compartments, which are loaded in an activation-dependent manner and translocated to the cell surface^{6–10}.

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In addition to MHC class I presentation of endogenous viral antigens following direct infection of DCs, mDCs are also capable of presenting exogenous antigens bound to class I molecules^{11,12}. This process, known as cross-presentation, is essential to the initiation of anti-viral immunity because DCs may not be directly targeted by all viruses, or an infecting virus may compromise DC function. The underlying mechanisms of cross-presentation remain an area of active investigation. Three primary models have been proposed. The first is an indirect pathway of exogenous antigen transfer from phago-endosomes to the cytosol for proteasomal processing and loading in the endoplasmic reticulum (ER) by the classical class I machinery¹³. The second is a direct pathway whereby antigen is processed and loaded on to class I entirely within the endosomal compartment, in a proteasome independent manner¹⁴. The third proposed model involves the delivery of ER components to endocytic organelles or the transport of incoming antigen to the ER^{15,16}, although the quantitative importance of this pathway remains unclear¹⁷.

Plasmacytoid DCs (pDCs) are a specialized subset of DCs central to the initiation of antiviral immunity, primarily through secretion of type I IFN^{18,19}. In addition to inhibiting viral replication, type I interferons activate the antiviral function of B cells, T cells, natural killer cells, and myeloid DCs²⁰. Thus, pDCs represent a critical connection between the innate and adaptive immune systems. Depletion of pDCs during viral infection leads to impaired CTL induction and viral clearance^{21,22}. A capability of pDCs as antigen presenting cells to directly stimulate T cell proliferation in response to viral infection however is less clear. Studies of murine and human pDCs have resulted in no clear consensus, some demonstrating little to no T cell stimulatory capacity²³, or a capacity equal to that of mDCs²⁴ and still others demonstrating an inhibitory role through the generation of regulatory T cells, depending on the time after activation pDC function was assessed^{25–27}.

RESULTS

pDCs drive CD8⁺ T cell proliferation

Healthy donor pDCs and mDCs were purified from blood by negative depletion followed by direct cell sorting for mDCs (HLA-DR high CD11c high) and pDCs (HLA-DR high CD123 high). Their APC function was assessed by their ability to induce the proliferation of allogeneic (allo) CD4⁺ and CD8⁺ T cells. pDCs demonstrated no allo-stimulatory capacity for either CD4⁺ or CD8⁺ T cells, while mDCs showed low stimulatory capacity (data not shown). Upon activation with influenza virus, both pDCs and mDCs induced strong proliferation of allo-CD4⁺ and CD8⁺ T cells. Influenza virus-treated pDCs induced stronger allo-proliferation of CD8⁺ T cells when compared to their myeloid counterpart (Fig. 1a,b, upper panels). Reactivation of the pDC-expanded CD8⁺ T cells with anti-CD3 and anti-CD28 led to high levels of IFN- γ secretion, indicating the acquisition of effector function (data not shown). In contrast, influenza virus-treated mDCs induced a higher proliferation of allo-CD4⁺ T cells (Fig. 1a,b, bottom panels) indicating that the difference in CD8⁺ T cell proliferation was not merely due to a maturation defect. Similar results were observed with a second, unrelated RNA virus (respiratory syncytial virus, data not shown).

pDCs rapidly cross present viral antigens

We then analyzed the ability of pDCs and mDCs to induce autologous viral antigen-specific CD8⁺ T cell expansion in HLA-A201⁺ donors. In the absence of viral exposure, no autologous CD8⁺ T cell proliferation was observed with either pDCs or mDCs (data not shown). Following 18 hr of influenza virus exposure, both DC subsets were able to induce CD8⁺ T cell expansion (Fig. 2a, upper panels). pDCs were more efficient at inducing expansion of CD8⁺ T cells in response to influenza virus. The proportion of antigen specific MP tetramer-specific CD8⁺ T cell expansion was also greater with pDCs (Fig. 2a). A 4 hr pulse with virus was sufficient for

pDCs to induce proliferation of influenza virus specific CD8⁺ T cells (Fig. 2a, bottom panels). pDCs were more effective than mDCs at inducing CD8⁺ T cell proliferation at this early time point. To exclude the possibility that influenza virus could continuously be processed and presented during the co-culture, pDCs and mDCs were fixed after a 6 hr pulse with influenza virus; fixed pDCs retained the ability to induce influenza MP-specific CD8⁺ T cell proliferation (Fig. 2b, left bottom panels). In contrast, fixed mDCs were incapable of stimulating either overall or MP-specific CD8⁺ T cell proliferation (Fig. 2b, right bottom panels). In addition, pDCs exposed to influenza virus were capable of priming allogeneic sorted naive CD8⁺ T cells although this priming event was considerably less potent than donor matched mDCs, indicating rapid pDC cross presentation leads primarily to activation of memory CD8⁺ T cells (Supplementary Fig. 1a,b online). Expansion of MP-tetramer positive cells was specific as no expansion was observed using the control HIV gag-tetramer, and the ubiquitous activation of total CD8⁺ T cells with anti-CD3 anti-CD28 microbeads did not result in a significant enrichment of MP-tetramer positive cells (Supplementary Fig. 2a,b online). Furthermore sorted CD8⁺ MP-tetramer positive T cells from pDCs cultures specifically recognize MP class I peptide complex presented on HLA-A201⁺ monocyte derived dendritic cells (Supplementary Fig. 2c online). As expected, pDCs did not produce type I interferon following fixation, (Supplementary Fig. 3 online) precluding a role for these molecules in directly influencing CD8⁺ T cell expansion. These data demonstrate that pDCs have the ability to cross present viral antigen within 6 hr. Thus pDCs are endowed with the capacity to rapidly elicit virus specific CD8⁺ T cell responses.

Influenza induces surface expression of MHC class I stores

The rapidity of the CD8⁺ T cell response suggested that presentation of viral antigens by pDCs may not require the synthesis of new MHC class I molecules but rather the loading of peptides onto a previously synthesized, intracellular pool. In the case of mDCs, it is well known that rapid presentation of exogenous antigens involves the loading of peptides on to MHC class II molecules found in late endosomes and lysosomes⁶. We next used confocal microscopy to determine if pDCs harbored an analogous intracellular pool of MHC class I. Although pDCs exhibited variable levels of surface class I, all immature pDCs show large perinuclear stores of intracellular MHC class I molecules (Fig. 3a). Intracellular stores were specific to pDCs, as donor matched immature mDCs demonstrated a reduced amount of MHC class I expression (Fig. 3a). Both DCs expressed intracellular stores of MHC class II molecules, with immature pDCs exhibiting a greater amount on the plasma membrane (Fig. 3a, bottom panel).

We next examined if pDC activation by influenza virus induced a redistribution of internal MHC class I to the cell surface. Exposure to the virus increased the surface expression of MHC class I (Fig. 3b). A significant increase was observed after only 30 min and continued for at least 4 hr (Fig. 3c,d and Supplementary Fig. 4 online). This rapid redistribution was specific for pDCs as donor matched mDCs showed no increase in surface MHC class I at this early time point (Supplementary Fig. 4a,b online). Pre-treatment of pDCs with the protein synthesis inhibitor cycloheximide (CHX) did not affect the increase in cell surface MHC class I on pDCs induced by a 30 min influenza virus exposure (Fig. 3e). Similarly, brefeldin A (which blocks ER to Golgi transport) also failed to inhibit the increase in surface MHC class I (Fig. 3e). Taken together, the modest but rapid increase in MHC class I at the surface, combined with its insensitivity to cycloheximide and brefeldin A, strongly suggest that activation of immature pDCs by virus causes at least a partial redistribution of MHC class I from intracellular sites to the plasma membrane.

pDCs store MHC class I within recycling endosomes

We next sought to determine the identity of the MHC class I-containing compartments. Although peptides are most typically loaded onto MHC class I molecules in the ER, the internal

MHC class I in pDCs was found not to co-localize with markers of the secretory pathway: calnexin (ER compartment), ERGIC-53 and COPII (ER-Golgi intermediate compartment), or GM130 (cisternal Golgi complex) (Fig. 4a). This result is consistent with our finding that the virus-induced increase in surface MHC class-I required neither new protein synthesis nor ER to Golgi transport. Some B cells, macrophages and immature DCs exhibit MHC class II molecules in late endosomal-lysosomal compartments where antigenic peptides are thought to be loaded onto MHC class II molecules²⁸. MHC class I has been reported in this compartment in a subset of mDCs^{29,30}. In immature pDCs, however, MHC class I did not co-localize with the late endosomal or lysosomal markers LAMP-1 or MHC class II (Fig. 4b).

The intracellular MHC class I did, however, appear to reside in some endocytic compartment. When immature pDCs were allowed to internalize influenza virus for 45 min, intracellular virus was found in the MHC class I-containing vesicles (Fig. 4c). Since early or recycling endosomes have been implicated in signaling by Toll-like receptors (TLR7,9) specific for viral nucleic acids, we determined if the MHC class I was located in the recycling endosomal compartment³¹. Indeed, in immature pDCs, intracellular MHC class I, but not MHC class II, exhibited a distribution similar to the recycling endosomal marker transferrin receptor (CD71) (Fig. 4d). Furthermore, internalized transferrin was also found to accumulate in class I-positive vesicles (Supplementary Fig. 5 online). This localization of MHC class I within the recycling endosomal compartment in pDCs suggests a mechanism by which MHC class I molecules may access exogenous antigen and translocate rapidly to the cell surface following viral stimulation. Furthermore these findings are consistent with published data that recycling class I molecules may play an important role in antigen cross-presentation in mice³².

Cross-presentation is independent of cytosolic transit

Cross-presentation of exogenous antigen in mDCs has been described as proteasome dependent¹³. To investigate the role of proteasome processing in pDCs MHC class I peptide formation, cells were treated for a prescribed period with specific inhibitors of proteolytic processing or endosomal trafficking, followed by 6 hour exposure to influenza virus (Fig. 5). Following activation, cell viability was assessed and cells were fixed, washed and incubated with autologous CD8⁺ T cells. Pretreatment of pDCs with a proteasome inhibitor, lactacystin, did not prevent presentation of influenza virus-derived antigen (Fig. 5a) in contrast to the marked inhibition obtained using chloroquine, an acidophilic weak base that inhibits endosomal acidification (Fig. 5b). Thus, the rapid generation of peptide MHC class I complex was proteasome-independent and dependent on endosomal acidification. Furthermore the formation of MHC class I peptide complex was sensitive to the cathepsin inhibitor FMK supporting a role for endosomal processing in complex formation (Fig. 5c). These results imply that the cross-presentation in pDCs is direct and does not require cytosolic intermediate step. In order to determine whether the intracellular stores of MHC class I localized in the recycling endosomal compartment correspond to the population of MHC class I presenting viral peptides, pDCs were pretreated with brefeldin A (BFA) to inhibit transport of *de novo* synthesized MHC class I. Presentation of MP peptide complex was insensitive to BFA pre-treatment, indicating that the processing observed in pDCs occurs in a post Golgi compartment and does not require contribution of *de novo* synthesized MHC class I for surface peptide complex formation (Fig. 5d). Formation of class I peptide complex was however sensitive to pretreatment with the recycling endosomal inhibitor primaquine³³, suggesting a role for class I localized to the recycling endosomal compartment in pDC cross-presentation (Fig. 5e). Vesicular processing was additionally supported by monitoring the presentation of inactivated virus by pDCs (Supplementary fig 6 online). These results were further confirmed by assessing cytokine (IFN- γ) production by a MP specific CD8⁺ T cell line (Fig. 5f). Chloroquine pretreatment of FLU-treated pDC completely inhibited IFN-g production by MP specific CD8⁺ T cell line while BFA pretreatment had no effect (Fig. 5f). The intravesicular nature of processing and

localization within the recycling endosomal compartment (Supplementary fig 7 online) could provide a mechanism for the rapid initiation of viral peptide specific CD8⁺ T cell expansion (observed in Fig. 2), because optimal cross-presentation via the cytosolic pathway in mDCs can take up to 18–24 hr³⁴.

DISCUSSION

Viral infections are characterized by rates of replication exceeding 10^{12} virions per day³⁵. It is imperative for the immune system to respond rapidly and specifically to this challenge. In humans, circulating pDCs are poor antigen presenting cells with an inability to induce either CD4⁺ or CD8⁺ T cell responses, and unlike mDCs, no phagocytic capacity³⁶. However following viral activation, pDCs undergo a radical transformation in both appearance and function, typified by the production of high levels of type I interferons^{37,38}. Here we have shown that immediately following viral activation, pDCs but not mDCs, are highly efficient at cross-presentation of viral peptides on MHC class I. Internalized virions or viral antigens are delivered to recycling endosomal compartments, which contain pre-synthesized MHC class-I molecules. MHC class I loading may occur within this compartment or in another endocytic compartment with the complexes accumulating in recycling endosomes before reaching the surface, as is true for a variety of recycling receptors. In any event, it seems unlikely that transport of viral antigens to the cytosol is required as pDCs were able to present inactivated virus and antigen presentation was independent of proteasome function. Within minutes of viral activation, MHC class I begins to translocate to the surface of the pDC suggesting that this pathway explains the rapidity and efficiency of pDC cross-presentation to CD8⁺ T cells. The recent description of functional Toll-like receptors present in these pDC endosomes suggests that these vesicles represent a self-contained specialized compartment for viral recognition, processing and cross-presentation^{31,39}. These findings not only reveal the central role of the pDC in the adaptive arm of viral immunity, but also provide insight into the basic mechanisms of cross-presentation. While this manuscript was under revision, Hoeffel *et al.* reported that pDCs were capable of efficiently cross-presenting lipopeptides as well as HIV antigens from apoptotic cells. This study did not identify the class I rich compartment that was identified herein and unlike our work, claimed that mDCs and pDCs may use the same pathway for cross-presentation⁴⁰. These distinctions may reflect differences in the kinetics of MHC class I presentation investigated and mechanisms of antigen delivery. Unlike Hoeffel *et al.*, we investigated cross-presentation that occurs almost immediately after virus entry thus demonstrating a unique feature of pDCs, namely the presence of a class I rich compartment which rapidly translocates to the cell surface following activation. It is the rate of generating these peptide MHC complexes which, in addition to the concurrent production of type I IFN, may play a key role in limiting viral replication. Early pDC cross-presentation may lead to rapid activation of peripheral anti-viral memory CD8⁺ T cell surveillance mechanisms. Later cross-presentation of infected cell bodies and viral debris by both pDCs and mDCs may play a critical role in both expanding the existing antiviral T cell population and priming new responses^{12,40}. However, this kinetically distinct cross-presentation event is likely to take place following migration from the site of infection to the draining lymph node, and have little consequence on the initial expansion of virus at the site of infection⁴¹.

The pDCs presents four remarkable cell biologic features to counteract viral infection: a similar shape to that of lymphocytes, for efficient trafficking to peripheral sites; an extensive ER compartment, to permit the prompt secretion of soluble factors, including type I interferons; an early endosomal compartment containing MHC class I molecules, the focus of this report, that appears to permit direct vesicular MHC class I loading for immediate activation of memory cytotoxic CD8⁺ T cells; and a late endosomal compartment containing MHC class II molecules, similar to that found in mDCs, which permits the rapid activation of CD4⁺ T cells. Thus, in both the MHC class I and class II pathways, pDCs can ensure a rapid response to viral infections

by utilizing pre-synthesized stores of MHC class I and II. Our studies demonstrate that in addition to their specialized role in the innate immune response to viruses, pDCs are uniquely capable of rapidly expanding viral antigen specific CD8⁺ T effector cells. Thus, pDCs are poised to control viral expansion, through nonspecific blockade of viral replication by type I IFN, and specific stimulation of antiviral cytotoxic CD8⁺ T cells. Strategies designed to target pDCs may form the basis of the next generation of anti-viral vaccines.

METHODS

Isolation of primary blood mDCs and pDCs

Studies using human cells were performed in accordance with the institutional guidelines of Baylor Research Institute and Yale University School of Medicine. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (Carter Blood Care, Grand Prairie, Tx) from healthy donors by Ficoll density gradient centrifugation. pDCs and mDCs were enriched with depleting unwanted cells using immunomagnetic beads coated with anti-CD3, CD14, CD16, CD56, CD19, and Glycophorin A (Stem Cell Technologies), according to manufacturer's protocol. Recovered cells were labeled with anti-HLA-DR-Quantum Red (Sigma Aldrich), Lineage (anti-CD3, CD8, CD14, CD19, CD20, CD56)-FITC, CD11c-APC, and CD123-PE (Becton Dickinson) and cells were sorted on a BD FACSAria. pDCs and mDCs were isolated based on their lack of lineage markers, and high expression of HLA-DR, and CD11c or CD123 (mDCs and pDCs, respectively). The purity of both DC subsets was $\geq 98\%$. For some imaging experiments, pDCs were isolated from elutriated monocytes from healthy blood donors using BDCA-4 magnetic beads and autoMACS sorting (Miltenyi) (purity $>90\%$).

pDC and mDC cell culture

Sorted peripheral blood pDCs and mDCs were cultured at 1×10^6 cells/ml in RPMI medium, 2 mM L-glutamine, penicillin/streptomycin, and 10% fetal calf serum. For MP-tetramer proliferation assay, both DC subsets were cultured in the presence of human serum. pDCs were cultured in the presence of IL-3 (10 ng/ml, R&D Systems), as survival factor. For DC activation, pDCs (in the absence of IL-3) and mDCs were cultured in the presence of live influenza virus A/PR/8/34 (40 HAU /ml; Charles River Laboratories) or UV inactivated influenza virus A/PR/8/34 (254nm cross linking for 20 minutes at maximum setting, UVGL-25 crosslinker, UVP). For some experiments, pDCs and mDCs were treated with Brefeldin A (BFA, 2.5 ug/ml), Cyclohexamide (CHX, 5 ug/ml), lactacystine (100 uM), primaquine (50 uM), chloroquine (50 uM) or Z-FA-FMK for 20 minutes before treatment (all compounds from Sigma).

Confocal microscopy

Immature pDCs and mDCs were cultured in 4 well-chamber slides (BD) and fixed in 4% paraformaldehyde for 20 minutes. Then, cells were permeabilized with 0.01% saponin in PBS and incubated with the following antibodies for 1 hr: anti-FITC conjugated MHC class I (w6/32 clone, eBioscience), FLU-NP (Abcam), Alexa 568 labeled- Phalloidin, -Calnexin (eBioscience), -COPII, -GM-130, -ERGIC 53 (from Ayano), -HLA-DM, -LAMP-1, -CD71 (all from Pharmingen) and -FITC or Alexa 568 labeled -HLA-DR (BD). Washed cells were mounted on to slides in Vecta Shield with DAPI media and imaged. Images were acquired either on a Leica TCS-NT/SPI Confocal Microscope with the 63 $\times$ Plan APO objective, using Leica TCS v 2.1227 software or a Zeiss LSM510 confocal microscope using a 100 $\times$ oil objective.

Immunophenotypic analysis

Cells were washed and resuspended in PBS containing 1% human AB serum, and incubated with FITC-conjugated class-I (w6/32 clone, eBioscience) antibody for 30 min at 4 °C. Samples were collected and analyzed using a FACSCalibur (Becton Dickinson) and data analysis was performed by CellQuest software (Becton Dickinson). Cells were electronically gated according to light-scatter properties, in order to exclude cell debris.

Mixed Lymphocyte Reaction (MLR)

Total allogenic CD4⁺ and CD8⁺ T cells were purified by negative selection using immunomagnetic beads (Stem Cell Technologies), according to manufacturer's protocol. T cell purity was ≥ 95%. CD4⁺ and CD8⁺ T cells were labeled with CFSE (1 μM, Molecular Probes) for 10 minutes and then, cultured with either IL-3 or influenza virus treated-pDCs or mDCs at ratio 1:20. For naive CD8 alloproliferation, naive CD8 were stained with CD27 and CD45RA antibodies and double positive CD8 were sorted on BD FACS Aria. Then, naive CD8 were stained with CFSE and cultured with pDCs or mDCs as described above. After 6 days, cells were collected, washed and stained with CD4-PE and CD8-APC conjugated antibodies (BD), according to T cell population. In order to exclude dead cells, recovered cells were resuspended in 7-AAD solution (20 μg/ml, Sigma) and analyzed on BD FACS Aria. Data analysis was performed using FlowJo software (Treestar).

Influenza MP-tetramer proliferation assay

CD8⁺ T cells were isolated from HLA-A2 donors as described above, and subsequently labeled with CFSE. Autologous IL-3 or influenza virus treated pDCs and mDCs were added to CD8⁺ T cells at ratio 1:20. For some experiments, pDCs and mDCs were fixed in cytochex (Dako) for 10 minutes and washed in 3 times in PBS before co-culture with the CD8⁺ T cells. After 6 days, cells were collected, washed and stained with anti- CD8-APC (BD) and Influenza Matrix Protein (MP) tetramer-PE (Beckman Coulter). In order to exclude dead cells, recovered cells were resuspended in 7-AAD solution (20 μg/ml) and analyzed on BD FACS Aria. Data analysis was performed using FlowJo software. To determinate the functionality of MP tetramer cell, MP tetramer positive CD8⁺ cells from FLU-treated pDCs were sorted and cultured in presence of allogenic HLA-A2⁺ GM-IL-4 DCs pulsed with IMF 58–66 peptide. After 18 hours, supernatants were collected and IFN-γ was measured by Luminex assay.

MP tetramer positive cell lines

HLA-A2⁺ PBMC were cultivated in RPMI medium complemented with 8% human serum in presence of 1 mM of IMF 58–66 peptide. IL-2 was added every two days. CD3, CD8 and MP tetramer positive were sorted and exposed to allogenic HLA-A2⁺ pDCs treated either with IL-3 or influenza virus in presence or absence of inhibitors. IFN-γ was measured by Luminex assay after 2 and 4 hours of culture.

Statistics

P-values were determined by the paired *t*-test using the Prism 4.0 (GraphPad).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Activated pDCs are powerful activators of allogeneic CD8⁺ T cells. **(a)** Flow cytometry of CFSE-labeled CD4⁺ or CD8⁺ T cells cultured for 6 days with allogeneic pDCs and mDCs activated with influenza virus for 18 hrs. Profiles of proliferating CD8⁺ (upper panels) and CD4⁺ (bottom panels) T cells are shown. Numbers indicate the percentage of proliferating T cells. **(b)** Same protocol as in **(a)** in 3 different matched donors. ($P = 0.03$, $n = 4$ pDCs vs mDCs/CD8⁺ T cells) and ($P = 0.02$, $n = 3$ pDCs vs mDCs/CD4⁺ T cells). Data are representative of at least three different matched donors in independent experiments.

Figure 2.

pDCs cross-present influenza virus antigen more rapidly and efficiently than mDCs. **(a)** Flow cytometric profiles of tetramer stained influenza-specific CD8⁺ T cells activated by pDCs and mDCs exposed to influenza virus for either 18 hrs (upper panels) or 4 hrs (bottom panels). pDCs and mDCs were then cultured for 6 days with autologous CFSE labeled CD8⁺ T cells. Numbers in the boxes represent percentage of MP tetramer positive proliferating CD8⁺ T cells. Numbers on left side indicate the percentage of total proliferating CD8⁺ T cells. Data are representative of three different experiments. **(b)** Same protocol as in **(a)** except pDCs and mDCs were treated with influenza virus and then fixed prior to 6 day culture with CFSE-labeled CD8⁺ T cells (bottom panels). Upper panels show the percentage of MP tetramer positive proliferating CD8⁺ T cells activated by unfixed 6 hr influenza virus treated pDCs and mDCs. Numbers in the boxes indicate percentage of MP-tetramer positive proliferating CD8⁺ T cells. Numbers on left side indicate the percentage of total proliferating CD8⁺ T cells. Data are representative of three different matched donors.

a

Figure 3.

MHC class I exists in large perinuclear intracellular pools within the pDCs and rapidly translocates to the cell surface after viral activation of pDCs. **(a)** Fluorescence microscopy of immature peripheral blood pDCs and mDCs stained with MHC class I (upper two panels) and

II (lower two panels) and phalloidin and then analyzed by confocal microscopy. Colocalization of staining was visualized in yellow in merged figures. **(b)** Flow cytometry of peripheral blood pDCs after influenza virus activation. Sorted pDCs (red line) were incubated in the presence of IL-3 (black line) or influenza virus (blue line) for 4 hrs and stained with anti-MHC class I antibody. Green line shows the isotype control staining. Data are representative of four different donors. **(c)** Kinetic of MHC class I surface expression on pDCs cultured in the presence of IL-3 (dashed line) and influenza virus (black line). Results are expressed as mean fluorescence intensity (MFI) and represent the mean $\pm$ s.d. of four different experiments. **(d)** Graphs of MHC class I surface expression MFI assessed after either 30 mins (left panel) ($n = 7$; $P = 0.009$) or 4 hrs (right panel) ($n = 4$; $P = 0.046$) of pDCs culture in the presence of either IL-3 or influenza virus. **(e)** pDCs were pre-treated with either BFA (right panel, $n = 7$; $P = \text{NS}$) or CHX (left panel, $n = 4$; $P = \text{NS}$) and cultured in the presence of influenza virus for additional 30 mins. Data are representative of at least four independent experiments.

a

b **c**

d **Figure 4.**

MHC class I is not localized in the traditional ER-Golgi transport pathway within pDCs. **(a–d)** Confocal fluorescence microscopy. **(a)** Colocalization of calnexin, ERGIC-53, COP-II and GM-130 with MHC class I in immature pDCs. **(b)** Colocalization of MHC class I with MHC class-II and LAMP-1 in immature pDCs analyzed. **(c)** Immature peripheral blood pDCs were exposed to influenza virus for 45 min, fixed and stained with antibodies to Flu-MP, and MHC class I. **(d)** MHC class I colocalizes with CD71. Immature pDCs were fixed, permeabilized and stained for MHC class I, MHC class II and CD71 (upper and bottom panels, respectively). Data are representative of three independent experiments.

f **Figure 5.**

Intracellular stores of MHC class I are localized in immature pDCs within the recycling endosomal compartment. (a–e) Dot plots show MP-tetramer CD8 proliferation after FLU-fixed pDC stimulation. pDCs are pre-treated with different inhibitors as shown in the correspondent plots and cultured for 6 hours with influenza virus. pDCs were then fixed and cultured for 6 days with CFSE-labeled CD8⁺ T cells. Left panels (untreated) show MP-tetramer positive CD8⁺ T cells stimulated with FLU-treated fixed pDCs in the absence of inhibitors. Right panels show results obtained in different matched donors. (f) IFN- γ secretion by MP specific cells after activation by FLU-treated fixed pDCs. MP specific cell lines are cultured with pDCs exposed to influenza virus in presence or absence of chloroquine and BFA inhibitors followed by fixation. As control, MP specific cell lines are stimulated by IL-3 pDCs. IFN-g is measured by Luminex assay. Results are expressed as the mean $\pm$ s.d. of three independent experiments.