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Antigen gene transfer to human plasmacytoid dendritic cells using recombinant adenovirus and vaccinia virus vectors

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Abstract. Recombinant adenoviruses (RAd) and recombinant vaccinia viruses (RVV) expressing tumour-associated antigens (TAA) are used as anti-tumour vaccines. It is important that these vaccines deliver the TAA to dendritic cells (DC) for the induction of a strong immune response. Infection of myeloid DC (MDC) with RAd alone is relatively inefficient but CD40 retargeting significantly increases transduction efficiency and DC maturation. Infection with RVV is efficient without DC maturation. Plasmacytoid dendritic cells (PDC) play a role in the innate immune response to viral infections through the secretion of IFNα but may also play a role in specific T-cell induction. The aim of our study was to investigate whether PDC are better targets for RAd and RVV based vaccines. RAd alone hardly infected PDC (2%) while CD40 retargeting did not improve transduction efficiency, but it did increase PDC maturation (25% CD83 positive cells). Accordingly, specific CTL activation by RAd infected PDC was limited (the number of IFNγ producing CTL was reduced by 75% compared to stimulation with peptide loaded PDC). RVV infected PDC specifically stimulated CTL but PDC were not activated. These results indicate that PDC are not ideal targets for RAd and RVV based vaccines. However, PDC induced specific CTL activation after pulsing with recombinant protein, indicating that PDC can also cross-present antigens released from surrounding infected cells.

Keywords: Human, plasmacytoid dendritic cells, antigen presentation/processing, vaccinia, adenovirus

Abbreviations

APC, antigen presenting cell; CAR, coxsackie-adenovirus receptor; GFP, green fluorescent protein; IFN, interferon; MDC, myeloid dendritic cell; MFI, mean fluorescence index; MoDC, monocyte derived dendritic cell; PDC, plasmacytoid dendritic cell; RAd, recombinant adenovirus; RVV, recombinant vaccinia virus; TAA, tumour associated antigen; TLR, toll-like receptor.

1. Introduction

Dendritic cells (DC) are the most potent antigen-presenting cell (APC) known to date and appear to be the only cell type capable of inducing primary T cell responses. DC are considered to be the sentinels of the body and play a key role in delivering antigen from the periphery to the secondary lymphoid organs. Precursors, originating from the bone marrow, migrate to virtually every organ in the body where immature DC take up surrounding antigens. After antigen uptake DC mature, co-stimulatory molecules (e.g. CD40, CD80, CD86) are up-regulated, *de novo* CD83 expression is induced and DC migrate to the lymphoid tissues where they activate effector T-cells [3]. However, it has become clear that DC function in tumor patients is often suppressed, probably due to tumor derived immunosuppressive factors [30]. Furthermore, malignant cells may not lead to immune activation due to the absence of inflammation and "danger" signals [33]. Apart from immunological silence, tolerance can also occur under these non-inflammatory circumstances due to antigen presentation by non-APC (the tumor cells) or due to improperly activated, tolerance-inducing APC [29]. Activation of tumor specific CTL by vaccination with activated DC may break both immunological silence and tolerance in cancer patients [10].

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Myeloid dendritic cells (MDC), characterized by CD11c expression are highly efficient antigen presenting cells capable of inducing primary T cell responses [3,13]. The introduction of methods to culture relatively large numbers of MDC ($CD14^+$ monocyte or $CD34^+$ progenitor cell derived) *in vitro*, have initiated MDC based vaccination trials for the treatment of cancer [2,28]. A second DC subset characterized by lack of CD11c and high levels of CD123 expression and by expression of the BDCA2 and BDCA4 markers [8] is the plasmacytoid dendritic cell subset (PDC) [12]. Myeloid DC and PDC differentially express toll like receptors (TLR). TLR are innate immune receptors that recognize conserved motifs on microbes and induce inflammatory signals. TLR4 is a receptor for LPS and is expressed on myeloid DC, while TLR9 is expressed on PDC [14]. Upon triggering of TLR9 on PDC by bacterial DNA high levels of IFNα are produced. Recognition of bacterial DNA is based on the presence of unmethylated CG dinucleotides in particular sequence contexts (CpG motifs). PDC are susceptible to stimulation by synthetic oligodeoxynucleotides that contain such CpG motifs (CpG ODN) through TLR 9 [19]. PDC also produce high levels of IFNα upon viral interaction, which has direct anti-viral effects and enhances cytotoxic effects of macrophages and Natural Killer cells. PDC infected by influenza virus stimulate allogeneic T-cells [16] and influenza specific T-cells [11]. PDC may not only play a role in eradicating viral infections but may also be of importance in eradicating virus induced tumors such as Human Papillomavirus induced cervical cancer [5].

Recombinant adeno (RAd) and vaccinia viruses (RVV) encoding tumor associated antigens (TAA) [6,18,31] have been used to deliver antigens to MDCs. RAd has been shown to have maturing effects on monocyte derived DC (MoDC) [32], but due to the lack of the adenovirus receptor CAR on MDC, high titers are necessary to obtain high transduction efficiencies *in vitro*. Titers can be reduced by using targeting approaches, which bypass the CAR and redirect the virus to antigens highly expressed on dendritic cells, such as CD40. This retargeting approach to CD40 also induces maturation of infected DCs [32]. RVV effectively infects MoDC but inhibits their maturation [9]. In this study we investigated whether PDC are better targets for these agents, and studied PDC activation/maturation as measured by IFNα production and CD83 expression as well as antigen presentation by PDC after RVV or RAd infection and cross-presentation after recombinant protein pulsing.

2. Materials and methods

2.1. DC isolation and culture

PBMC were isolated from buffy coats of normal human volunteer donors by density centrifugation over Lymphoprep (Nycomed AS, Oslo, Norway). PDC were isolated (>90% pure as shown by BDCA2 and CD123 double staining) using the $BDCA4^+$ cell MACS isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's recommendations. The mean yield was 6.9×10^5/buffy coat (range 3–9 $\times$ 10^5). Isolated $BDCA4^+$ cells were cultured at 3–5 $\times$ 10^5 cells per ml in Yssels medium supplement with 2% human pooled serum and 10 ng/ml IL-3 (R&D systems, Abingdon, Oxon, UK). MoDC of the same donor were generated as described previously [4].

2.2. Stimulation, infection and peptide loading of DC

$BDCA4^+$ PDC were incubated for 24 hours with 3 μg/ml CpG2216 (a generous gift of Art Krieg, Coley Pharmaceutical Group, MA) as a positive control for PDC activation. Infection with adenovirus type 5 (100 pfu/cell), encoding GFP (RAd-GFP, kindly provided by Tanja D. de Gruijl, VUMC, Amsterdam), influenza M1 protein (RAd128 a generous gift from Dr. Rickards and Dr. Wilkinson, University of Wales, UK) or HPV 16 E6/E7 (RAd101, kindly provided by Dr. S. Inglis, Xenova, Cambridge, UK) all under a CMV promotor, was done using either lipofectamine or the anti-CD40/anti-fiber knob chemical conjugate (CC, kindly provided by Tanja D. de Gruijl, VUMC, Amsterdam) as previously described [4,32]. Vaccinia virus (v1114) infection: DC were washed once in serum free medium and incubated for 4 hours at 37°C with 20 pfu/cell of either the wild type vaccinia virus (RVV-wt) or vaccinia virus encoding influenza M1 protein (RVV-M1).

Peptide loading with the HLA-A2.1 binding influenza M1 derived peptide ($M1_{58–66}$) or the HLA-A2.1 binding HPV16 E7 derived peptide ($E7_{11–20}$) as a negative control (25 μM each) was done for 2–4 hours at room temperature in serum free medium in the presence of β2-microglobulin (3 μg/ml). For cross-presentation experiments DC were allowed to take up recombinant influenza M1 protein (100 μg/ml, a generous gift from Sjoerd van der Burg, LUMC, Leiden) at 4°C or 37°C. After 4 hours the cells were washed and incubated o/n at 37°C in an incubator with 5% CO_2 hu-

midified atmosphere. All infections, stimulations and peptide loading were done in the presence of 10 ng/ml IL-3 for the PDC and in the presence of 1000 U/ml IL-4 (Centraal Laboratorium Bloedbank, Amsterdam, The Netherlands) and 100 ng/ml GM-CSF (Schering-Plough, Madison, NJ) for the MoDC.

2.3. Flow cytometry

Cells were incubated at 4°C for 30 min in PBS with 0.1% BSA and 0.01% NaN_3, in the presence of appropriate dilutions of FITC- or PE-labeled isotype controls and mouse mAbs to BDCA-2 and BDCA-4 (Miltenyi Biotec, Bergisch Gladbach, Germany), CD123, CCR7 (Pharmingen, San Diego, CA), CD83 (Immunotech, Marseille, France), $\alpha v\beta 3$ (Chemicon, Temecula, CA), $\alpha v\beta 5$ (Life technologies, Gaithersburg, MD) and coxsackie adenovirus receptor, CAR (a generous gift from Robert W Finberg, Harvard Medical School, Boston, MA). A second incubation step was performed for the unconjugated mAbs with FITC-labeled goat anti-mouse Abs (Centraal Laboratorium van de Bloedtransfusiedienst, Amsterdam, The Netherlands) or goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL). The cells were subsequently analyzed, using a FACS Calibur and CellQuest FACS analysis software (BD Biosciences, Erembodegem-Aalst, Belgium).

2.4. IFNα ELISA

IFNα production was measured in supernatants of stimulated and infected DC (5×10^5/ml) using the human IFNα ELISA kit (Biosource, Nivelles, Belgium) according to the manufacturer's recommendations.

2.5. IFNγ ELISPOT

An HLA-A2.1 restricted influenza M1 specific ($M1_{58–66}$) CTL clone [27] (1000/well) with infected and stimulated DC as target cells (1000/well) were used in an IFNγ ELISPOT assay (Mabtech, Nacka, Sweden) as previously described [4].

2.6. DEX-FITC uptake

DC were incubated for 1 hour with 1 mg/ml of dextran-FITC (MW 70,000; Molecular Probes, Leiden, The Netherlands) on ice or at 37°C. Free dextran-FITC was washed away and uptake was determined by flow cytometry. The ratio of the mean fluorescence intensity of DC incubated at both temperatures was a measure of active dextran-FITC uptake.

2.7. Statistics

The amount of IFNα produced by PDC after different stimuli were compared using a two-sided Student's T-test.

3. Results

3.1. Low transduction efficiency of PDC by RAd

PDC and MoDC were infected with RAd-GFP with or without an anti-CD40/anti-fiber knob chemical conjugate. It has previously been shown that this conjugate, which blocks binding of the fiber knob to its natural receptor CAR, increases adenoviral transduction of MoDC. This was confirmed by our results showing an increased transduction of MoDC by the conjugate (17% versus 92% GFP positive cells). In contrast to MoDC, PDC could hardly be transduced either with or without the conjugate (1% versus 2% GFP positive cells, Fig. 1a). The fiber knob of the adenovirus binds to the CAR while either of the αv integrins; $\alpha v\beta 3$ or $\alpha v\beta 5$ is a necessary co-receptor for internalization of the virus. MoDC lack CAR expression but do express $\alpha v\beta 5$ [32] ensuring internalization of the virus after transduction in the presence of the anti-CD40/anti-fiber knob chemical conjugate which bypasses the CAR. However, only a low percentage of PDC express CAR (3%), $\alpha v\beta 3$(3%) and $\alpha v\beta 5$ (7%) (Fig. 1b) explaining the low transduction efficiency even in the presence of the conjugate.

3.2. RAd and RVV do not induce phenotypic PDC maturation, but RAd induces a low level of IFNα production by PDC

RVV and RAd did not induce phenotypic maturation of PDC, illustrated by a lack of CD83 and CCR7 up-regulation. Moderate PDC maturation was only induced by the combination of the conjugate and RAd-GFP (25% CD83 positive cells). The conjugate alone and in combination with RAd-GFP induces a strong maturation of MoDC (74% and 73% CD83 positive cells, respectively). CpG2216 induced CD83 expression on about a third of the MoDC and PDC (Fig. 1c). RVV, CC, RAd or the combination of RAd and CC did not induce CCR7, which is necessary for migration to the lymph nodes, while CpG2216 did induce CCR7 expression on PDC (not shown). PDC activation was measured by IFNα production after incuba-

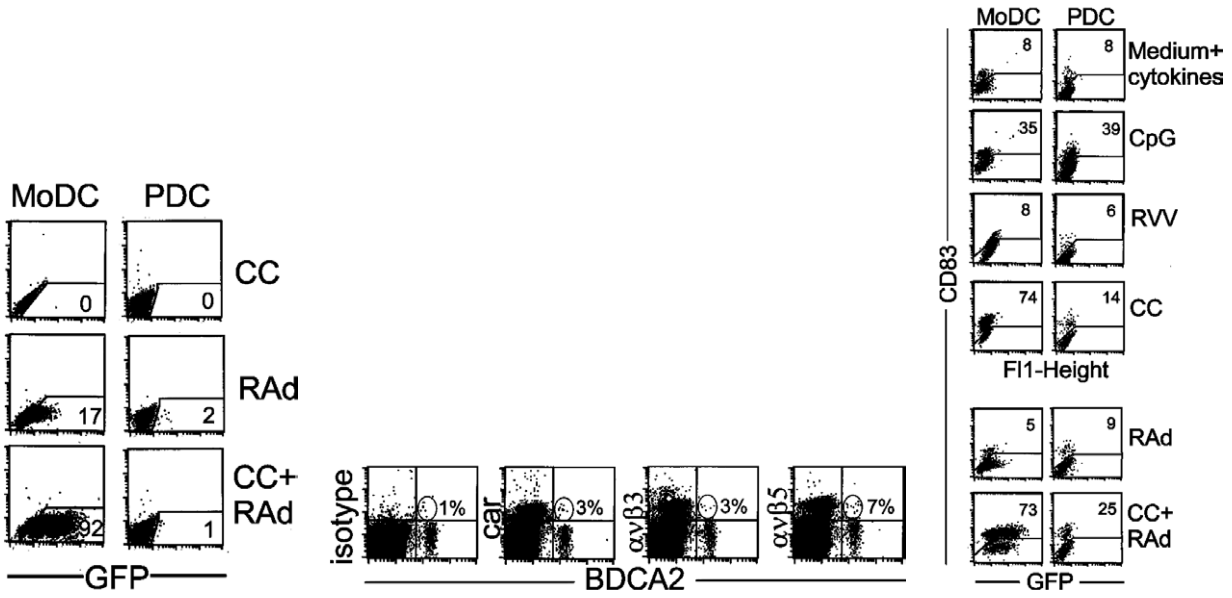

Fig. 1. (a) MoDC and PDC were incubated with the bi-specific chemical conjugate of anti-CD40 and anti-fiber knob mAb (CC) alone, RAd-GFP alone or the combination of CC and RAd-GFP. The numbers indicate the percentage of GFP positive cells. (b) Total PBMC were stained with BDCA2 in combination with antibodies directed against CAR, $\alpha v\beta 3$ and $\alpha v\beta 5$. The numbers indicate the proportion of BDCA2 positive cells that express these (co)-receptors (c) MoDC and PDC were incubated with medium, CpG2216, RVV, CC alone, RAd-GFP alone and CC and RAd-GFP combined. The numbers indicate the percentage of CD83 positive cells.

tion with the various agents. Low levels of IFNα production (mean 1.9 ng/ml) were induced by RAd infection alone ($p = 0.029$ compared to medium control) and in combination with the conjugate to similar levels while the conjugate alone and RVV did not induce IFNα production by PDC (Fig. 2a). However, the levels of IFNα induced by RAd were significantly lower compared to the levels induced by CpG2216 (mean 43 ng/ml; $p = 0.016$). On the other hand, MoDC did not produce IFNα upon any of the stimuli given (not shown). These results show that no or only a low level of PDC activation is induced by RVV and RAd, respectively.

3.3. PDC present endogenous antigens delivered by RAd and RVV

PDC were compared with MoDC for their capacity to process and present the influenza M1 antigen delivered by RAd and RVV to an HLA-A2.1 restricted $M1_{58–66}$ specific CTL clone. The DC were loaded with either a control peptide or the M1 peptide, infected with RAd encoding either HPV16 E6/E7 (control) or the M1 protein, or infected with wild type RVV or RVV encoding M1. Peptide loaded and infected DC were incubated overnight in an anti-IFNγ coated ELISPOT plate in the presence of the M1 specific CTL clone. Peptide loaded PDC induced antigen specific IFNγ release by the CTL clone (Fig. 2b). Despite the low transduction efficiency (Fig. 1a), and low (co)-receptor expression (Fig. 2b) RAd infected PDC also induced antigen specific IFNγ release by the CTL clone. MoDC induced a much stronger response, most probably due to higher transduction efficiency of MoDC by RAd. RVV infected PDC induced antigen specific IFNγ release by the CTL clone to similar levels as RVV infected MoDC (Fig. 2b). In conclusion, RVV and to a limited extend RAd deliver antigens to PDC, which are processed and presented to $CD8^+$ T-cells.

3.4. PDC present exogenous antigens to antigen specific $CD8^+$ CTL

Subsequently we tested whether PDC were able to take up and present exogenous antigen. PDC were capable of endocytosis as shown by their capacity to take up dextran-FITC. However, MoDC take up dextran-FITC more efficiently as illustrated by the higher MFI (Fig. 3a). PDC were capable of cross-presentation, as

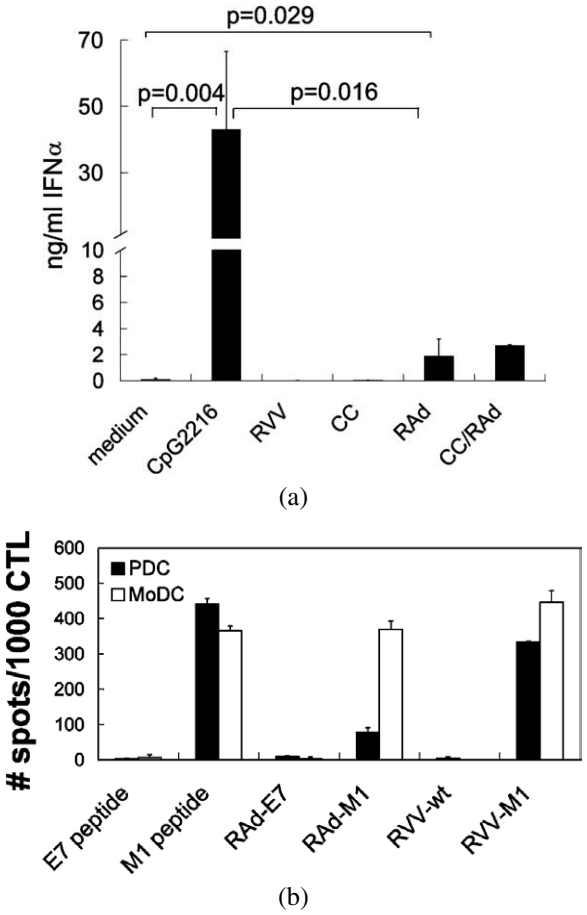

Fig. 2. (a) IFNα production by PDC after incubation with medium, CpG2216, RVV and RAd with and without CC. P-values (Student's T-test) indicate significant differences in IFNα production between the indicated conditions. (b) IFNγ ELISPOT assay of M1 specific CTL clone stimulated with peptide loaded and RAd and RVV infected PDC and MoDC. The number or specific spots per 1000 CTL are shown.

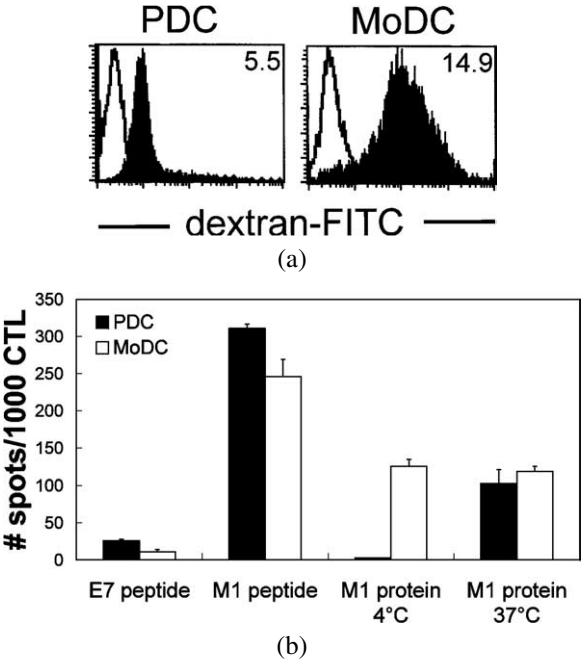

Fig. 3. (a) Analysis of dextran-FITC uptake by PDC and MoDC. The ratio of the mean fluorescence intensity of cells incubated with dextran-FITC at 37°C and cells incubated on ice is given (mean fluorescence index, MFI). (b) IFNγ ELISPOT assay of M1 specific CTL clone stimulated with PDC loaded with recombinant M1 protein. The number or specific spots per 1000 CTL are shown.

shown by the antigen specific IFNγ release by the CTL clone after stimulation with PDC, which were fed with recombinant M1 protein. PDC fed at 4°C were used as negative controls (Fig. 3b). In conclusion, PDC are capable of processing and cross-presenting exogenous antigens, comparable to MoDC.

4. Discussion

Recombinant adenovirus is one of the most commonly used vectors for gene transfer into MDC, although clinical studies are limited [24]. Our results confirmed a modest transduction by RAd alone and a high transduction and maturation of MoDC by an anti-CD40/anti-fiber knob chemical conjugate in combination with RAd-GFP [32]. In contrast, PDC maturation was not induced by RAd alone, while the CD40 conjugate and in particular the conjugate in combination with RAd had a modest effect on maturation. This indicates that RAd increased cross-linking of the CD40 conjugate and that the maturation we observe is due to CD40 ligation rather than RAd stimulation and/or infection. Furthermore, RAd infects PDC with an extremely low efficiency both with and without the chemical conjugate. Since our PDC preparations were $>$90% pure (result not shown) we cannot exclude the presence of contaminating myeloid DC. Blood derived myeloid DC are infected, although with low efficiency, by adenovirus type 5 [22] and infected MDC may be responsible for the low level of GFP expression after RAd-GFP infection and low level of CTL activation after RAd-M1 infection of isolated PDC. However, Fig. 1b shows that in total PBMC a low percentage of cells positive for BDCA2, which is a specific marker for PDC and is not expressed on MDC, also express adenovirus (co)-receptors, indicating that a small proportion of PDC is susceptible to adenovirus infection. Furthermore, PDC secrete high amounts of

IFNα, whereas monocytes and MDC produce little or undetectable amounts of IFNα [17,21]. Figure 2a shows that isolated PDC produce a substantial amount of IFNα upon RAd stimulation, indicating that PDC are activated by RAd to produce IFNα. MoDC lack TLR9 expression and are therefore not expected to be activated by CpG [19], however Fig. 1c shows an up-regulation in CD83 expression after CpG stimulation. This could either be due to an indirect effect of IFNα production by contaminating PDC and/or NK cells in the MoDC cultures [23], although we never detected any IFNα production after CpG stimulation of MoDC. Another possible explanation could be trace amounts of endotoxins in the CpG preparation that can trigger TLR4, which is expressed on MoDC but not on PDC [14].

RVV is another vector used in cancer vaccination trials [6,18]. Transduction efficiency of PDC by RVV, appears to be much more efficient than RAd as suggested by the strong activation of the CTL clone by RVV infected PDC. However, our data show, as has also been shown for MoDC, that RVV does not induce PDC maturation, possible due to the ability of RVV to inhibit host cell protein synthesis, or due to the expression of proteins that interfere with the antiviral effects of IFNα [1,20]. Induction of primary tumor specific CTL depends on mature DC presenting TAA. Our data therefore indicate that PDC are not suitable APC for RVV and RAd based vaccines aiming at induction of tumor specific CTL, due to lack of transduction and/or activation.

Notably, we show here that human PDC are also capable of cross-presenting antigen, which is an important natural method of antigen presentation [7]. PDC and MoDC pulsed with antigen at 37°C induced a similar level of CTL activation. However, there was CTL activation induced by MoDC loaded with protein at 4°C. This was probably due to residual protein adhered to the cell surface, which was internalized and processed during co-culture with the CTL at 37°C. PDC loaded at 4°C did not induce CTL activation indicating differential protein adhesion and uptake by PDC and MoDC, which is also illustrated by the differential uptake of dextran-FITC. These results indicate that PDC are capable of taking up surrounding antigens and cross-presenting them to CTL, however it is not yet clear whether this holds true for all proteins. Since the up-take is mediated by (receptor)binding to the cell, different proteins may be differentially taken up by PDC.

Although the primary function of PDC will be the inhibition of viral replication [16] and induction of plasma cell differentiation [15,25] through IFNα production, our results and those of others [11,26] indicate that stimulation of antigen specific T cells is an additional property of PDC. Whether PDC are suitable antigen presenting cells to also prime CTL, not only by converting naive MART-1 specific CTL precursors into effector CTL [26], but also by inducing tumor antigen specific CTL that are undetectable before priming, remains to be investigated.

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