Functional comparison of DC generated in vivo with Flt3 ligand or in vitro from blood monocytes: Differential regulation of function by specific classes of physiologic stimuli


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Abstract

Dendritic cells (DC) are a family of leukocytes that initiate T and B cell immunity against pathogens. Migration of antigen-loaded DC from sites of infection into draining lymphoid tissues is fundamental to the priming of T cell immune responses. In humans, the major peripheral blood DC (PBDC) types, CD1b/c+ DC and IL-3R+ plasmacytoid DC, are significantly expanded in vivo using Flt3 ligand (FL). DC-like cells can also be generated from monocyte precursors (MoDC). A detailed comparison of the functional potential of these types of DC (in an autologous setting) has yet to be reported. Here, we compared the functional capacity of FL-expanded CD1b/c+ PBDC with autologous MoDC in response to three different classes of stimuli: (1) pro-inflammatory mediators, (2) soluble CD40L (CD40L) and (3) intact bacteria (E.coli). Significant differences in functional capacities were found with respect to changes in phenotype, migratory capacity, cytokine secretion and T cell stimulation. MoDC required specific stimuli for the expression of functions. They responded vigorously to CD40L or E.coli, expressing cytokines known to regulate IFN-γ in T cells (IL-12p70, IL-18 and IL-23) but required PGE₂ during stimulation to migrate to chemokines. In contrast, PBDC matured in response to minimal stimulation, rapidly acquired migratory function in the absence of PGE₂-containing stimuli and were low cytokine producers. Interestingly, both types of DC were equivalent with respect to stimulation of allogeneic-T cell proliferation and presentation of peptides to CTL lines. These distinct differences are of particular importance when considering the choice of DC types for clinical applications.
Introduction

Dendritic cells (DC) are rare bone marrow-derived cells, involved in antigen capture, processing and presentation. DC are uniquely able to prime a naïve T cell response.\textsuperscript{1,2} Because of their critical role in orchestrating the immune response, there is increasing interest in utilizing DC as cellular vaccine adjuvants in the immunotherapy of cancer.\textsuperscript{3,4} A range of soluble factors and pathogen signals are known to activate DC.\textsuperscript{1,2} Thus the maturation state of vaccine-loaded DC will likely be critical for their regulation of appropriate T cell immune responses. Three main sources of DC have been used in clinical trials, derived from: (i) CD34\textsuperscript{+} progenitor cells; (ii) CD14\textsuperscript{+} monocytes and (iii) peripheral blood DC precursors. Generation of CD34\textsuperscript{+}-derived DC requires 10-28 days \textit{in vitro} culture,\textsuperscript{5,6} whilst DC generated \textit{in vitro} from CD14\textsuperscript{+} monocytes (MoDC) require 5-7 days.\textsuperscript{7,8} Although their physiological relevance \textit{in vivo} remains unclear, MoDC are the major DC type used in vaccine-based clinical studies.\textsuperscript{3,4} MoDC have also been used to establish many of the biologic paradigms of DC function.\textsuperscript{1,2} An alternative, and perhaps more physiological, source of DC in humans are the immature DC populations found in peripheral blood.\textsuperscript{9,10} At least two PBDC populations, constituting less than 1\% of total mononuclear cells, exist in human peripheral blood: CD1b/c\textsuperscript{+} PBDC and IL-3R\textsuperscript{+} plasmacytoid DC (PDC).\textsuperscript{9-13} Several cytokines are known to expand the number of these PBDC types \textit{in vivo}, including G-CSF and Flt-3 ligand (FL).\textsuperscript{9,14,15} FL expands both human CD1b/c\textsuperscript{+} PBDC and IL-3R\textsuperscript{+} PDC\textsuperscript{9,10,14-18} and has anti-tumor effects in animal models.\textsuperscript{19-21} It has been suggested that the CD1b/c\textsuperscript{+} PBDC subset in peripheral blood is related to the CD14-derived dermal DC and to germinal center DC.\textsuperscript{6,22,23} Both of these types of DC appear to be of myeloid origin and can differentiate into Langerhans cells in the presence of TGF-\beta.\textsuperscript{12,24} However, little is currently known of the functional differences between the CD1b/c\textsuperscript{+} PBDC and MoDC types (e.g. antigen uptake capacity, migration, cytokine secretion and regulation of T cell function).

Few direct comparisons between DC types have been reported. Comparisons of CD34\textsuperscript{+}-derived DC and MoDC suggest that CD34\textsuperscript{+}-derived DC may be superior at activating low frequency, peptide-specific cytotoxic T lymphocytes.\textsuperscript{25-28} Others have reported that IL-3R\textsuperscript{+} PDC are functionally different from MoDC.\textsuperscript{13,29-36} However, few of these studies have directly compared DC functions in an autologous setting, most comparing DC types between
allogeneic donor sources. Thus, the degree to which donor-to-donor variation contributes to the observed functional differences may be significant.

We performed a clinical trial that evaluated FL (to expand PBDC numbers) with or without peptide vaccination in patients with malignant melanoma. The present study describes the functional analysis of FL-expanded CD1b/c+ PBDC isolated from these patients and compares them with autologous MoDC. Furthermore, CD1b/c+ PBDC and autologous MoDC from healthy donors were also compared to exclude the possibility that functional differences between DC types from cancer patients were due to the cancer itself or alterations in DC behavior due to FL administration. We found major differences between the responses of MoDC and CD1b/c+ PBDC towards three different classes of physiologic stimuli with respect to migratory function, cytokine production and regulation of T cell function.
Materials and Methods

Media
DC were cultured in RPMI 1640 (Trace Biosciences, Melbourne, Australia) supplemented with 20mM HEPES, 60mg/L penicillin G, 12.6 mg/L streptomycin, 2mM L-glutamine, 1% non-essential amino acids and 10% heat-inactivated fetal calf serum (FCS) (CSL Limited, Melbourne, Australia) in a 5% CO₂ incubator. Mixed leukocyte reactions (MLR) were performed in Iscoves Modified Dulbecco Medium (IMDM) (GIBCO/Life Technologies, NY) with 5% pooled normal human serum (gift of the Victorian Tissue Typing Service, Royal Melbourne Hospital, Australia) in a 10% CO₂ incubator.

Monoclonal antibodies and cytokines
Flow cytometric analysis of DC and T cells was performed using the following monoclonal antibodies: FITC-conjugated IgG1 isotype control; PE-conjugated IgG1 isotype control; anti-CD1a, anti-CD1c; anti-CD1d; anti-CD45RA; anti-CD80; anti-CD83; anti-CD86; anti-CD123 (IL-3Rα); anti-HLA-DR; anti-macrophage mannose receptor (MMR); anti-CXCR3, anti-CD3, anti-CD8, anti-IFN-γ (all from PharMingen/Becton Dickinson, San Jose, CA); anti-CCR6 (R&D systems, Minneapolis, MN); anti-blood dendritic cell antigen (BDCA)-2 and anti-BDCA-3 (Miltenyi Biotech, Auburn, CA). The following recombinant human cytokines were added to DC cultures: TNF-α (10 ng/mL), IL-4 (500 U/mL) (both Peprotech Inc, Rocky Hill, NJ), GM-CSF (40 ng/mL) (Schering-Plough, Sydney, Australia) and IFN-α2a (1000 IU/mL) (Roferon-A, Roche Products Pty Ltd, Sydney, Australia). PGE₂ (1µM final concentration) was purchased from Sigma Chemical Company (St Louis, MO). Soluble CD40L-trimer (CD40L) (1 µg/mL final concentration) was a kind gift from Amgen Inc. (Seattle, WA).

Cell sources
CD1b/c⁺ PBDC and monocytes were isolated from peripheral blood mononuclear cells (PBMC) of patients with stage II, III or IV melanoma enrolled in a phase I clinical study (LUD97-012) receiving 14 consecutive days of FL (Amgen Inc.) (25 µg/kg/day) alone or in combination with peptide vaccines; or from buffy packs from healthy donors and provided by the Australian Red Cross Blood Bank (Southbank, Melbourne). In the present study, the various types of DC were examined from patients with minimal residual disease to exclude
the possible issues of advanced cancer on decreasing the functional capacity of DC via the release of immunosuppressive cytokines. Blood for monocyte isolation was taken prior to administration of FL, and on day 15 for CD1b/c⁺ PBDC isolation. The Protocol Review Committee of the Ludwig Institute for Cancer Research and the Human Research Ethics Committee of the Austin & Repatriation Medical Centre approved the protocol, and informed consent was obtained from all patients.

CD14⁺ monocytes were affinity purified using the MACS CD14 isolation kit (Miltenyi Biotech, Auburn, CA) and cultured (7 days) in RPMI / 10% FCS (5x10⁵/mL) with GM-CSF (40 ng/mL) and IL-4 (500 U/mL) in 24-well plates to generate MoDC (>95% of cultured cells). On day 7, all wells were pooled and re-adjusted to a concentration of 5x10⁵ DC per mL. Maturation-inducing factors were added on day 7 and cells and supernatants were harvested on day 8 or 9 for functional assessment. Cytokines and other stimuli in the present study (e.g. TNF-α, IFN-α2a, CD40L, PGE₂ and intact E.coli) were titrated and the concentrations used in the figures represent those found to be optimal.

**Enrichment of CD1b/c⁺ PBDC from FL-treated patients and healthy volunteers**

CD1b/c⁺ PBDC were enriched from frozen PBMC samples obtained from the clinical trial (LUD-97-012). After thawing, CD14⁺ monocytes, CD19⁺ B cells and CD3⁺ T cells were depleted using immunomagnetic beads (MACS, Miltenyi Biotech, Auburn, CA) according to the manufacturer’s instructions. This depletion procedure routinely yielded >60% CD1b/c⁺ CD14⁺ HLA-DR⁺ PBDC as assessed by FACS. The enriched PBDC were then stained with anti CD1b/c-FITC (Biosource Limited, Camarillo, CA), anti-CD123-PE (IL-3Rα) and anti-HLA-DR-APC (both PharMingen/Becton Dickinson) and sorted as a CD1b/c⁺, CD123lo, HLA-DR⁺ population on a MoFlo cell sorter (94-98% purity) (Cytomation, Fort Collins, USA). Sorted CD1b/c⁺ PBDC were then cultured in 24-well plates (5x10⁵/well) in RPMI/10% FCS for 2 days with various combinations of stimuli prior to assessment of function. In some experiments CD1b/c⁺ PBDC and autologous CD14⁺ monocytes were positively selected using magnetic bead isolation. PBMC were sequentially treated with anti-CD14 beads MACS, Miltenyi Biotech, Auburn, CA) and CD14⁺ monocytes (>96% purity) cultured in GM-CSF and IL-4 for the generation of MoDC. The residual PBMC were then incubated with anti-BDCA-1 (anti-CD1c) beads (MACS, Miltenyi Biotech, Auburn, CA) and CD1b/c⁺ PBDC isolated (>97% purity).
Cell migration assay
Assays were performed as previously described. Briefly, lower chambers of Transwell plates (8.0 µm pore size) (Costar, Corning, NY) were filled with 500 µl RPMI/10% FCS with or without chemokines: CCL21 (MIP-3β) (300ng/mL), CCL19 (6Ckine) (100ng/mL) or CXCL12 (SDF-1α) (30ng/mL) (all from Peprotech Inc, Rocky Hill, NJ). DCs (1-2x10⁴) were added in 50µL of RPMI/10%FCS into the upper chamber. After 2 hours, cells in the lower chambers were harvested, concentrated to 50 µL volumes in Eppendorf tubes and counted microscopically with a hemocytometer. Each stimulation condition was performed in triplicate wells.

RNA isolation and cDNA synthesis
Total RNA was isolated from MoDC and CD1b/c⁺ PBDC using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. In brief, cells were lysed and homogenized in lysis buffer containing guanidine isothiocyanate and β-mercaptoethanol. Seventy percent ethanol was added to the samples, the RNA immobilized on spin columns and eluted in RNase-free water. 0.16 µg total RNA was used to synthesize cDNA using 1 µg random hexamers (Promega, Madison, WI), 1 mM dNTP’s (Amersham Pharmacia Biotech, Piscataway, NJ), 2 units RNAse inhibitor (Promega), 5 mM MgCl₂ (Applied Biosystems, Foster City, CA), 1x polymerase chain reaction (PCR) Buffer (Applied Biosystems) and 2 units M-MLV reverse transcriptase (Life Technologies, Rockville, Maryland) in a 20µl reaction, for 60 minutes at 42°C. The enzyme was inactivated at 95°C for 5 minutes. One µl of the resulting 20µL cDNA was used for “real time” PCR quantitation.

Quantitative Real Time PCR
Pre-developed assay reagents (PDAR's) for IL-12p35, IL-12p40 and IL-18 were obtained from PE Applied Biosystems and used in multiplex reactions with 18S rRNA PDAR (PE Applied Biosystems) for normalization. Primers and probe for IL-23p19 were designed using Primer Express software, version 1.5a (PE Applied BioSystems). Gene expression levels were quantitated using ABI Prism 7700 Sequence Detection System (Applied Biosystem). PCR reactions were set up in 96 well plates (25 µl/reaction) according to the manufacturer’s instructions and analyzed using the SDS program, version v1.7 (PE Applied BioSystems).
Relative expression was calculated using the ΔCt method and is expressed relative to a calibrator, in this case the GM-CSF/IL-4 DC control as previously described.\textsuperscript{38}

\textit{Cytokine ELISAs}

Cytokine secretion by stimulated DC or by allogeneic T cells was measured by cytokine ELISAs. Cytokine ELISA kits were purchased for IL-2, IL-5, IL-6, IFN-α, IL-10, IL-12p70 (Opteia, PharMingen/Becton Dickinson, San Diego, CA), IL-18 (MBL, Nagoya, Japan) and PGE\textsubscript{2} (BioScientific, NSW, Australia). Capture and HRP-conjugated detection antibodies for IFN-γ ELISAs were a kind gift from CSL (Melbourne, Australia). PGE\textsubscript{2}, IFN-α, IL-6, IL-10, IL-12p70, IL-18 and IFN-γ were performed on supernatant (SN) from DC cultures and IFN-γ, IL-2, IL-5 and IL-10 ELISAs were performed on SN from allo-MLRs according to the manufacturer’s instructions using Maxisorp plates (Nunc, Denmark). The horseradish peroxidase (HRP)-substrate was tetramethylbenzidine (TMB) peroxidase (KPL, Gaithersburg, Maryland); the color reaction was terminated by adding 100 µL ortho-phosphoric acid (1 M). Plates were read in a Thermomax microplate reader (BioMediq, Australia).

\textit{T cell purification and Mixed Leukocyte Reaction}

Allogeneic CD\textsuperscript{2+} T lymphocytes were obtained by rosetting PBMC with aminoethylisothiouronium (AET)-treated sheep red blood cells. T cells were between 88-95% pure based on CD3 staining. Varying numbers of DC were cultured in round-bottomed 96-well plates in triplicate with 10\textsuperscript{5} allogeneic PBMC for 5 days in IMDM with 5% human serum. After 5 days, 200 µL of supernatants were harvested and fresh medium containing 1 µCi/well \textsuperscript{3}H-thymidine (DuPont, Sydney, MA) was added for 8 hours. Cells were transferred onto a glass fiber filter (Wallac, Turku, Finland), and \textsuperscript{3}H-thymidine-incorporation was measured using an NXT TopCount Betaplate scintillation counter (Packard, Meriden, CT). In separate experiments the CD\textsuperscript{2+} T cells (1x10\textsuperscript{6}) were labeled with CFSE (0.01mM) in serum-free PBS in the dark (10 min at RT). T cells were then washed and cultured (3x10\textsuperscript{5}) with immature or mature MoDC (1x10\textsuperscript{4}) in round-bottomed 96-well plates in triplicate for 5 days. On day 5, cultures were re-stimulated with freshly matured MoDC in the presence of 10 µg/mL Brefeldin A at 37°C for 8h. Cells were harvested, pelleted, stained with anti-CD8-APC and CD3-Cy-Chrome (PharMingen/Becton Dickinson), washed again, then fixed with 1% paraformaldehyde (ProSciTech)/Phosphate buffered saline (PBS) before staining with FITC.
conjugated anti-IFN-γ (PharMingen/Becton Dickinson)/0.2% saponin/PBS at 4°C overnight. Cells were then analyzed using FACS.

**DC-peptide presentation to a CTL line**

6 x 10^{-6} to 6 x 10^{-12} M of the HLA-A2-restricted peptides NY-ESO-1b (amino acids 157-165, sequence SLLMWITQC, Biological Production Facility, Ludwig Institute for Cancer Research, Melbourne, Australia) and EBV (BMLF1 sequence amino acids 280-288, GLCTLVAML, Austin Research Institute, Melbourne, Australia) were treated at RT for 1 hour with 500 µM Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) (Pierce) in cystine-free Dulbecco’s Modified Eagle medium (Cys-free DMEM) (Gibco) to reduce dimerized peptides to monomeric form. MoDC or CD1b/c^{+} PBDC or the TAP-deficient T2 cells were resuspended in Cys-free DMEM and equal volumes were added to the reduced peptide and pulsed at room temperature for 30 minutes. The DC or T2 cells were then washed once and resuspended in RPMI/10% FCS and 10 µg/mL Brefeldin A at a concentration of 1x10^{6} cells/mL. 100 µL peptide-pulsed DC or T2 cells were incubated with 100 µL of peptide-specific T cells (APC:effector ratio of 1:1) at 37°C for 4 hours in a 96 well U-bottom plate. Cells were pelleted, stained with anti-CD8 Cy-Chrome, washed, then fixed with 1% paraformaldehyde (ProSciTech)/Phosphate buffered saline (PBS) before staining with FITC conjugated anti-IFN-γ/0.2% saponin/PBS at 4°C overnight. Cells were then analyzed using FACS.
Results

Cell morphology and culture of MoDC and CD1b/c+ PBDC

CD1b/c+ PBDCs were purified from the PBMC of melanoma patients (with minimal residual disease) treated with FL by removal of lineage positive cells by mAb-MACS bead depletion and cell sorting of lineage negative cells on the basis of CD1b/c and HLA-DR expression to greater than 97% purity. CD1b/c+ PBDCs showed poor viability if cultured in medium alone but this was substantially improved when cultured with GM-CSF and IL-4. FL-expanded CD1b/c+ PBDCs were morphologically identical to their counterparts from untreated individuals with a typical multi-lobulated nuclear morphology (Figure 1, panel A). To avoid issues relating to the myelopoietic effects of FL upon monocyte development in vivo,\(^9\) the present study generated autologous MoDC from CD14+ monocytes isolated from blood samples taken prior to FL administration. Immature MoDC (GM-CSF+IL-4) were morphologically distinct from freshly isolated CD1b/c+ PBDC, being larger with round or kidney-shaped nuclear morphology and more extensive cytoplasm (Figure 1, panel B). Both FL-expanded, CD1b/c+ PBDC and MoDC displayed typical morphologic features of mature DC following stimulation with CD40L, including prominent dendritic processes (Figure 1, panels C and D).

Phenotypic analysis and maturation of MoDC and CD1b/c+ PBDC

Freshly isolated, CD1b/c+ PBDC were phenotypically immature, expressing low levels of the maturation markers CD80, CD83 (Figure 2A) and CD86 (data not shown). Consistent with previous reports, CD1a was constitutively expressed on MoDC but was not present on freshly isolated PBDC (Figure 2A). In contrast, CD1b/c+ PBDC but not MoDC expressed CD1d. Both DC types expressed CD1c (Figure 2A) as well as CD1b, CD11c, CD13, CD33 and CD54 (data not shown), consistent with a putative myeloid origin. Whereas CD1b/c+ PBDC spontaneously up-regulated the expression of CD80, CD83 and CD86 following overnight culture in GM-CSF and IL-4-containing medium,\(^39\) MoDC required maturation with specific combinations of stimuli.\(^39\) As previously reported, FL-expanded CD1b/c+ PBDC showed heterogeneous expression of MMR.\(^9\) Additionally, discrete subpopulations within the CD1b/c+ PBDC gate were also detected on the basis of CCR6 and BDCA-3 expression (Figure 2B). The percentage expression for MMR, CCR6 and BDCA-3 suggests that multiple
subpopulations are likely to exist. Interestingly, CD1b/c+ PBDC up-regulated surface expression of CD83 (Figure 2C) and HLA-DR (data not shown) more rapidly than MoDC, regardless of maturational stimulus. Furthermore, the mean fluorescence intensity of these markers was an order of magnitude greater for CD1b/c+ PBDC compared to MoDC (Figure 2C).

**Induction of cytokine secretion by highly purified CD1b/c+ PBDC and MoDC**

DC produce several types of cytokines following stimulation with pathogen or CD40L, such as IL-6, IL-10, IL-12p70. We compared cytokine secretion by CD1b/c+ PBDC and MoDC in response to different classes of physiological stimuli. Figure 3A shows that MoDC secreted considerably more IL-6 compared to CD1b/c+ PBDC, particularly in response to *E.coli*. Similarly, MoDC secreted IL-10 in response to a range of stimuli, but produced the highest levels of IL-10 following stimulation with *E.coli* (Figure 3B). As previously reported, the addition of PGE2 to CD40L or to *E.coli* decreased the amount of IL-10 produced by MoDC.38

IL-12p70 is critical for the induction of IFN-γ production by T cells. Bioactive IL-12p70 is composed of two subunits (IL-12p35 and IL-12p40). Another IL-12 family member, IL-23, has overlapping effects with IL-12p70 and is composed of IL-12p40 and the novel IL-23p19 subunit. We evaluated the expression of IL-12p70 and IL-23 by CD1b/c+ PBDC and autologous MoDC in order to evaluate the potential of these DC subpopulations to induce T cell IFN-γ production. Figure 3C shows that MoDC are potent producers of IL-12p70, especially following stimulation with *E.coli*, whereas CD1b/c+ PBDC are poor IL-12p70 producers, confirming previous results.38,39

**Induction of cytokine secretion by CD1b/c+ PBDC following initial in vitro culture prior to stimulation.**

Previous reports, as well as this study, indicate that freshly isolated CD1b/c+ PBDC are relatively poor producers of cytokines following immediate stimulation.38,39 However, two studies have shown that CD1b/c+ PBDC can produce IL-12p70 following *in vitro* stimulation. In both studies, PBDC were initially cultured (thus matured) for at least 24h prior to stimulation with LPS or CD40L.18,40 We, therefore, evaluated whether *in vitro* maturation of CD1b/c+ PBDC enhanced their responsiveness to IL-12p70-inducing stimuli such as CD40L, intact *E.coli* or the combination of IL-1β, IFN-γ, CD40L and *E.coli*. It was found that
increased production of IL-12p70 was observed in response to the combination of GM-CSF, IL-1β, IFN-γ, CD40L and E.coli (Figure 3D) but not GM-CSF+CD40L nor GM-CSF+E.coli (data not shown). Figure 3D also shows that CD1b/c+ PBDC required prolonged in vitro culture (24-48h) prior to stimulation in order to produce increased levels of IL-12p70 (~400pg/ml). Shorter times of in vitro-maturation (2-24h) prior to stimulation were not sufficient at enhancing IL-12p70 producing capacity. However, even under these optimized conditions, IL-12p70 production by CD1b/c+ PBDC was consistently lower than by MoDC. Furthermore, Figure 3D also demonstrates that the low production of cytokines by CD1b/c+ PBDC shown in Figures 3A-C was not due to the attenuating effects of IL-4 since similar levels of IL-12p70 were produced regardless of whether IL-4 was present or absent from the stimulation cocktail.

Using quantitative real time PCR (qRT-PCR), we examined whether the low levels of IL-12p70 produced by CD1b/c+ PBDC are due to low levels of IL-12p35 or IL-12p40 mRNA expression. Figure 4A and 4B demonstrate that for MoDC, the levels of IL-12p35 mRNA expression correlated with IL-12p70 production by ELISA. Similarly, for CD1b/c+ PBDC, we found that low IL-12p70 secretion correlated with low expression of IL-12p35 and IL-12p40 mRNA (Figure 4A and B). Finally, the novel IL-23p19 mRNA was neither constitutively expressed by freshly isolated CD1b/c+ PBDC nor induced following stimulation. In contrast, immature MoDC constitutively expressed IL-23p19 mRNA and this was further increased following stimulation with E.coli (Figure 4C).

IL-18, like IL-12p70 and IL-23, can induce IFN-γ secretion by T cells. Production of IL-18 by CD1b/c+ PBDC or MoDC was investigated. Figure 4D shows that immature MoDC (GM-CSF+IL-4) constitutively produced low levels of bioactive IL-18 (~50pg/ml) and that secretion was increased upon stimulation with either CD40L or E.coli (Figure 4D). In contrast, CD1b/c+ PBDC were poor producers of bioactive IL-18 irrespective of the type of stimulus encountered.

Analysis of migratory capacity of MoDC and CD1b/c+ PBDC
Migration of antigen-loaded DC toward lymphoid organs is critical for the initiation of T cell immunity and requires the expression of the chemokine receptor, CCR7, to respond to the lymph node-directing chemokines, CCL19 (MIP-3β or ELC) or CCL21 (6Ckine or SLC). The migratory capacity of CD1b/c+ PBDC and autologous MoDC in response to CCL21 was
assessed next. PGE$_2$ is a critical regulator of migratory function in MoDC.$^{38,41}$ The addition of PGE$_2$ reduced the ability of either CD40L or $E. coli$ to induce cytokine secretion in MoDC (Figure 3A-C) whilst at the same time inducing MoDC migratory function (Figure 5C).$^{38,41}$ In contrast, PGE$_2$ was less critical at regulating these functions in CD1b/c$^+$ PBDC, which spontaneously migrated following maturation with all the classes of stimuli irrespective of the presence of PGE$_2$ (Figure 5D).$^{38,41}$ We next examined whether the kinetics with which CD1b/c$^+$ PBDC acquired migratory function paralleled that of MoDC. As shown, CD1b/c$^+$ PBDC (Figure 5B and D) acquired migratory capacity in vitro more rapidly (8h) compared to autologous MoDC (24h) (Figure 5A and C). The differing kinetics regarding acquisition of migratory function between FL-generated CD1b/c$^+$ PBDC and autologous MoDC were also seen with DC from healthy individuals (data not shown).

It has been proposed that MoDC depend upon exogenous PGE$_2$ as a consequence of IL-4 blocking endogenous PGE$_2$ production by immature MoDC.$^{42}$ Alternatively, CD1b/c$^+$ PBDCs, which are efficient migratory cells in the absence of PGE$_2$-containing stimuli may secrete higher levels of PGE$_2$ in culture and thus not depend upon exogenous PGE$_2$ to acquire migratory function. To address these possibilities, we examined the levels of PGE$_2$ produced in culture SN by the two DC types. As shown in Figure 5E, both MoDC and CD1b/c$^+$ PBDC constitutively secreted comparable levels of PGE$_2$ in vitro and these levels were further increased following stimulation with $E. coli$. Although not conclusive, these data argue that the differences in migratory capacity between MoDC and CD1b/c$^+$ PBDC are not simply due to differences in the endogenous production of PGE$_2$.

Comparison of T cell stimulatory capacity of MoDC and CD1b/c$^+$ PBDC
Mature DC are the most efficient stimulators of naïve T cells. We investigated the relative ability of differentially matured CD1b/c$^+$ PBDC or autologous MoDC to stimulate the proliferation and cytokine secretion of allo-reactive T cells in a mixed leukocyte reaction (MLR). CD1b/c$^+$ PBDC and autologous MoDC were equally effective in stimulating allo-T cell proliferation (Figure 6). However, MoDC required prior activation with various physiological stimuli to induce maximal T cell proliferation. In this regard, immature MoDC (GM-CSF+IL-4) were, on a per cell basis, 10-100 times less efficient at inducing T cell proliferation than mature MoDC (Figure 6). In contrast, CD1b/c$^+$ PBDC induced equivalent T cell proliferation to that seen with MoDC irrespective of the stimulation conditions. This is
consistent with the fact that CD1b/c+ PBDC fully mature in culture without the need for further stimulation.

**T cell proliferation and cytokine secretion induced by MoDC and/or CD1b/c+ PBDC**

Next we assessed DC-mediated cytokine secretion by allo-reactive T cells in a separate series of experiments. Induction of IFN-γ by CD4+ T cells was assessed by intracellular cytokine secretion (ICS) using immature or matured MoDC. Here, T cell proliferation could be examined in parallel by labeling CD3+ T cells with CFSE prior to co-culture with DC for 5 days. After 5 days stimulation, T cell proliferation and IFN-γ secretion was assessed by FACS analysis by gating on CD3+ CD8- T cells during analysis. Similar functional profiles were noted for CD8+ T cells (data not shown). The majority of CFSE-labeled T cells cultured in the absence of MoDC died over the course of the culture. The few surviving T cells maintained much of their CFSE levels indicating that little T cell division occurred in the absence of stimulation with APC (data not shown). As shown in Figure 7, immature MoDC (GM-CSF+IL-4) were poor stimulators of CD4+ T cell division as well as IFN-γ secretion. MoDC that secreted the highest levels of IL-12p70 (i.e. those matured with CD40L or E.coli) also induced the highest proportion of IFN-γ-secreting CD4+ T cells (12% and 20% respectively). Furthermore, not all IFN-γ-producing CD4+ T cells had maximally divided based on proportion of T cells with low CFSE labeling. In contrast, migratory-type MoDC (TNF-α+IFN-α+PGE2) induced few CD4+ T cells to secrete IFN-γ (1-2%), with the majority of these maximally dividing based on reduction of CSFE labeling. Finally, although CD40L+PGE2-matured MoDC induced fewer IFN-γ-producing CD4+ T cells (1.5%), E.coli+PGE2-matured MoDC remained potent inducers of T cell IFN-γ (14.8%) as compared to MoDC matured with E.coli alone (20%)(Figure 7).

The ability of MoDC and CD1b/c+ PBDC to stimulate T cell cytokine secretion was also assessed by measuring IL-2, IL-5 and IFN-γ in MLR culture SN by ELISA. MoDC were more potent inducers of T cell cytokines than autologous CD1b/c+ PBDC, inducing T cells to secrete higher levels of IL-2, IL-5 and IFN-γ (Figures 8A-C). Once again, stimuli that induced maximal IL-12p70 and/or IFN-γ production by MoDC (i.e. CD40L or E.coli) correlated with their capacity to induce the highest levels of IFN-γ by T cells (Figure 8C). Furthermore, MoDC matured with TNF-α, IFN-α and PGE2 induced higher levels of IL-2 and IL-5
production in allo-reactive T cells (Figure 8A and B). Interestingly, MoDC matured with \textit{E. coli}+PGE\textsubscript{2} expressed a mixed functional profile; that is MoDC with efficient migratory capacity (Figure 5A and C) and induction of high levels of IFN-\(\gamma\) by T cells (Figure 8C). Finally, despite negligible production of IL-12p70 or IFN-\(\gamma\) by CD1b/c\(^+\) PBDC, these DC did induce IL-2, IL-5 and IFN-\(\gamma\) by allogeneic T cells, albeit less efficiently than autologous MoDC (Figure 8A-C).

\textit{Presentation of synthetic peptide to CTL lines by MoDC and CD1b/c\(^+\) PBDC}

Finally, in order to assess antigen presentation to T cells, different populations of DC were used in a peptide-Ag presentation assay. In this assay, peptide-specific T cells were induced to produce IFN-\(\gamma\) following co-culture with peptide loaded DC. The peptides tested were HLA-A2-restricted peptides derived from the tumor-associated antigen, NY-ESO-1 (NY-ESO-1\(_{\text{b157-165}}\)) and the viral antigen, EBV BLMF-1 (BLMF-1\(_{\text{280-288}}\)). Short-term CTL lines were generated (2-5% peptide-specific as assessed by peptide tetramer analysis) following culture of PBMC for 7-10 days with the respective peptides and used as responders in the assays. As shown in Figure 9, both CD1b/c\(^+\) PBDC and autologous MoDC were equivalent, at the per cell level, to TAP-deficient T2 cells at presenting NY-ESO-1\(_{\text{b157-165}}\) and EBV BLMF-1\(_{\text{280-288}}\) to peptide-specific CTL lines as assessed by intracellular IFN-\(\gamma\) staining. Importantly, both DC types could present peptides at between the \(10^{-7}\) and \(10^{-9}\) Molar range, indicating that both types of DC were efficient at presenting limiting concentrations of peptide to CTL lines.
Discussion

The clinical application of DC requires a detailed understanding of their functional potential and how best to manipulate this for optimal vaccine delivery and immune induction. Both PBDC and MoDC are currently being evaluated in anti-cancer immunotherapy trials (see reviews3,4,43). This study provides the first detailed, direct comparison of these two DC populations by comparing autologous DC types under identical conditions. Both FACS-sorted CD1b/c+ PBDC and highly pure MoDC were isolated from melanoma patients (with minimal residual disease) participating in a clinical trial evaluating FL as a vaccine adjuvant.37 Both DC types were cultured in the same media, containing GM-CSF and IL-4 for optimal viability.7,29 A variety of cancers have been shown to affect the generation of functionally mature MoDC.44,45 Indeed, we found that MoDC and PBDC from patients with later stage, metastatic disease receiving FL expressed reduced functional capacities such as the ability to mature in response to in vitro stimulation and ability to stimulate T cells. Furthermore, some of these patients expressed significant monocytosis following FL treatment as well as elevated serum levels of pro-inflammatory cytokines such as IL-6.37 However, MoDC and CD1b/c+ PBDC used in the present studies were specifically derived from patients with minimal residual disease and these DC were found to be functionally similar to their counterparts from healthy individuals (references38,39,46 and data not shown). Several important findings were made in the present study. First, CD1b/c+ PBDC and autologous MoDC are phenotypically and functionally distinct DC differing in their migratory ability and their capacity to secrete specific cytokines, including IL-6, IL-10 and IL-12p70. Second, the function of these two DC subtypes was regulated by different types of soluble mediators. Finally, although these two DC types were equivalent at presenting peptides and T cell stimulation, they induced different levels of T cell cytokines.

Both MoDC and CD1b/c+ PBDC are frequently considered to be similar cell populations.13,29-36 Although, the phenotype of these two distinct DC subtypes is similar, (e.g. expression of CD4 and the myeloid markers CD11c, CD13 and CD33) there are several markers that distinguish them. For instance, MoDC express CD1a but not CD1d, whereas CD1b/c+ PBDC express CD1d but not CD1a. In addition, whilst the majority of MoDC express the pattern recognition receptor, MMR, only a subset (8-15%) of freshly isolated CD1b/c+ PBDC expressed MMR. In this regard, CD1b/c+ PBDC appear to be phenotypically heterogeneous,
composed of distinct subsets expressing surface Ags not expressed on immature MoDC (e.g. CCR6 and/or BDCA-3). The percentage of CD1b/c+ PBDC expressing MMR, CCR6 or BDCA-3 suggests that multiple subpopulations likely exist. It is unclear, however, whether these markers define distinct subsets or represent the same PBDC population at different stages of maturation. We, and others, have noted that freshly-isolated FL-mobilized PBDC are immature cells that rapidly mature (CD80+, CD83+, CD86+) following culture.10,16,39,47 The present study also indicates that this occurs with greater amplitude than for MoDC. Although MoDC and CD1b/c+PBDC express a similar repertoire of pathogen recognition receptors (e.g. MMR, DEC20548 and toll-like receptors30,49), MoDC produce higher levels of IL-1β, IL-6, IL-10 and IL-12p70 in response to pathogen signals.31,49

Several cytokines can induce IFN-γ in T cells including, IL-12p70, IL-23 and IL-18. Bioactive IL-12p70 is a heterodimeric cytokine composed of an inducible IL-12p35 subunit and a constitutively expressed IL-12p40 subunit. IL-12p40 can also homodimerize to form IL-12(p40)2, a putative “antagonist” of IL-12p70 function,50 or heterodimerize with a recently identified IL-23p19 subunit to form a novel cytokine, IL-23, which has overlapping biological effects with IL-12p70.51 Furthermore, IL-18 has been shown to synergise with IL-12 and induce T cell IFN-γ.52 Although others have reported that human DC may express IL-18 mRNA,23,53 there are no reports of differential expression between MoDC and CD1b/c+PBDC. We have shown that CD1b/c+PBDC are poor producers of IL-12p70 whereas MoDC are prolific producers of this cytokine.38,39 The present study demonstrates that the differences in production of bioactive IL-12p70 between MoDC and CD1b/c+PBDC are reflected at the gene level, with CD1b/c+PBDC expressing negligible IL-12p35 and p40 mRNA (even following stimulation) compared with MoDC. Furthermore, in contrast to MoDC, CD1b/c+PBDC did not express IL-23p19 mRNA (either constitutively or following stimulation), nor did they secrete bioactive IL-18 following stimulation. Interestingly, two reports indicate that CD1b/c+PBDC can produce high levels of IL-12p70.18,40 Both studies initially cultured the CD1b/c+PBDC in GM-CSF prior to stimulation. The present study confirms the need for maturation prior to stimulation but shows that: (a) CD1b/c+PBDC require at least 24-48h maturation prior to stimulation and (b) that significant IL-12p70 is only induced by the combination of IL-1β, IFN-γ, CD40L and E.coli. However, even under these optimized conditions, CD1b/c+PBDC were still lower producers of IL-12p70 compared to MoDC. It is unclear whether the prolonged in vitro culture step enhanced sensitivity of PBDC to inducers
of IL-12p70 or induced their differentiation into MoDC-like cells. Omission of IL-4 from the CD1b/c⁺ PBDC culture conditions did not enhance stimuli-induced IL-12p70 secretion suggesting that the potential attenuating effects of IL-4 are not the reason for the low cytokine secreting capacity of PBDC following stimulation. In any case, these data indicate that MoDC have the potential to produce at least three cytokines known to induce IFN-γ in T cells (IL-12p70, IL-18 and IL-23), whereas CD1b/c⁺ PBDC were poor producers of these cytokines. This likely reflects the reduced ability of CD1b/c⁺ PBDCs to induce T cell cytokine production in vitro. Interestingly, Osada et al. have reported IL-12-independent-induction of T cell IFN-γ by PBDC suggesting that PBDC can produce a yet unidentified IFN-γ-inducing factor(s). 54

Although MoDC and CD1b/c⁺ PBDC express a similar repertoire of chemokine receptors, 55-57 chemokine receptor expression by MoDC is not predictive of their migratory function. 38,41 Major differences in migratory capacity were observed between these two DC types. CD1b/c⁺ PBDC migrated to chemokines shortly after culture (8-12h), this requiring only minimal in vitro manipulation (e.g. GM-CSF and IL-4), whereas MoDC required prolonged culture (24h) with PGE₂-containing stimuli. PGE₂ appears to regulate MoDC migratory function via cAMP/protein kinase A activation. 38,39 Interestingly, migratory-type MoDC (i.e. matured with PGE₂-containing stimuli) exhibit a similar functional profile to CD1b/c⁺ PBDC (i.e. migratory, low IL-12p70 production and induction of IL-2 by T cells). PGE₂ has also been shown to abrogate IL-12p70 secretion by MoDC. 58,59 Zelle-Rieser et al. also implicate IL-4-mediated suppression of endogenous PGE₂ production by MoDC for the maturation-enhancing effects of exogenous PGE₂. 42 However, we found that PGE₂ production by immature MoDC (GM-CSF+IL-4) was comparable to levels produced by CD1b/c⁺ PBDC and that both DC populations increased PGE₂ production following stimulation. Thus, differences in the endogenous levels of PGE₂ between CD1b/c⁺ PBDC and MoDC cannot completely explain their different migratory capacity. An alternative is that CD1b/c⁺ PBDC are the product of a completely distinct stimulation history in vivo to MoDC. In particular, cyclic AMP (cAMP) analogs, which can replace the ability of PGE₂ to induce MoDC migration, 38 are present in serum (e.g. vasoactive intestinal peptide 60 or sympathomimetics 61 ). It is possible that freshly isolated CD1b/c⁺ PBDC have already been exposed to cAMP-inducing serum factors in situ and thus are pre-sensitized to migrate upon minimal in vitro stimulation. Work to address these specific questions is ongoing.
Analysis of the ability of each DC type to induce T cell function revealed that MoDC and CD1b/c<sup>+</sup> PBDC were equivalent at inducing allo-reactive T cell proliferation and were as efficient as TAP-deficient T2 cells at presenting peptides to CTL lines. As reported, however, MoDC required prior maturation with specific stimuli to induce efficient T cell stimulation,<sup>54,62</sup> whereas CD1b/c<sup>+</sup> PBDC (that spontaneously mature in vitro without additional stimuli) efficiently induce T cell stimulation. Major differences were also observed in the type and quantity of cytokines the DC populations induced T cells to secrete, this paralleling differences in IL-12p70 production by the DC types. Stimuli that maximally induced IL-12p70 (e.g. CD40L and E.coli) preferentially skewed T cells toward the production of IFN-γ (Figure 7 and 8C). In contrast, PGE<sub>2</sub>-containing stimuli (e.g. TNF-α+IFN-α+PGE<sub>2</sub> or CD40L+PGE<sub>2</sub>) induced lower levels of IFN-γ and increased IL-2 and IL-5 production by T cells. The one exception was the combination of E.coli+PGE<sub>2</sub>. This combination resulted in MoDC expressing a mixed functional profile. That is, migratory type MoDC (Figure 5A and C) that also secreted high levels of IFN-γ (Figure 3A) and induced high levels of IFN-γ by T cells (Figure 7 and 8C). This suggests that migratory function and cytokine secretion can be co-expressed by MoDC in the context of pathogen signals and that this class of stimulus can override some of the attenuating affects of PGE<sub>2</sub> upon MoDC cytokine secretion. Interestingly, E.coli-derived LPS was, in our hands, a sub-optimal stimulus at inducing these functional changes in MoDC, compared to the intact E.coli pathogen (data not shown). This likely reflects the more complex array of pattern recognition receptors that intact E.coli would be recognized by (e.g. Toll-like receptors 2, 4, 6 and C-type lectins) as compared to LPS which would be recognized through the TLR-4/CD14 complex (reviewed by Medzhitov<sup>63</sup>).

Our clinical trial found that FL expanded the number of immature CD1b/c<sup>+</sup> and IL-3R<sup>+</sup> PBDC in patients with melanoma.<sup>37</sup> However, discernible immune responses were infrequent and clinical responses rare. Similarly, a recent study suggested that vaccines using FL as an adjuvant did not enhance T-cell proliferative responses but did increase the precursor frequency of IFN-γ secreting HER-2/neu-specific T cells.<sup>47</sup> In contrast, Fong et al. demonstrated immunological and clinical responses using FL-mobilized PBDC pulsed with a CEA-derived peptide ex vivo.<sup>10</sup> The ex vivo enrichment and culture step was noted to induce
PBDC maturation, again highlighting the importance of the maturational state of DC upon the T cell immune outcome. A recent review emphasizes that immature DC may induce T-cell tolerance or anergy. If true for immature PBDC, then the work of Fong and results presented here suggest that *ex vivo* maturation of FL-generated PBDC may enhance their immune potency and minimize their potential to dampen immune response induction. The present study provides crucial information to optimally manipulate PBDC *in vitro* in order to produce cells with defined functional characteristics. Given that not all DC types nor all stages of their maturation will be appropriate for the initiation of immune responses, it is critical that one matches the most appropriate type or stage of DC with the clinical aim at hand.
Figures

**Figure 1. Morphology of immature and mature MoDC and CD1b/c⁺ PBDC.**

PBDC were purified by negative depletion from the peripheral blood of patients treated with FL for 14 consecutive days and then FACS sorted to high purity (>95%) on the basis of CD1b/c and HLA-DR expression. Autologous MoDC were generated from blood taken prior to FL administration and cultured for 7 days prior to the parallel isolation of PBDC. MoDC were prepared by culturing purified CD14⁺ monocytes for 7 days in GM-CSF and IL-4. A. Freshly sorted CD1b/c⁺ PBDC. B. Immature MoDC. C. CD1b/c⁺ PBDC stimulated (2d) with CD40L. D. MoDC stimulated (2d) with CD40L. Figures are representative of > 10 experiments. All photomicrographs x100 magnification.
Figure 2. Surface marker expression of freshly isolated and matured CD1b/c⁺ PBDC.

A. Surface antigen expression of maturation markers on CD1b/c⁺ PBDC. Isotype control is marked by the broken-lined, unfilled histogram; surface staining of PBDC is shown by filled histograms. B. Surface expression of the chemokine receptor CCR6, blood dendritic cell antigen-3 (BDCA-3) and macrophage mannose receptor (MMR). Crosshairs reflect background settings based on the isotype-matched Ab controls. Data are representative of three separate experiments. C. Surface expression of CD83 on CD1b/c⁺ PBDC and MoDC at 2h, 12h and 18h following stimulation with either GM-CSF and IL-4 alone or in combination with TNF-α, IFN-α and PGE₂ or with CD40L.
Figure 3. Secretion of cytokines by immature and mature MoDC and CD1b/c⁺ PBDC.

Immature MoDC (GM-CSF+IL-4) or freshly sorted CD1b/c⁺ PBDC were stimulated for 2 days with either TNF-α,IFN-α,PGE₂ or CD40L ± PGE₂ or *E.coli* ± PGE₂ and cytokine ELISAs performed on culture supernatant. A. IL-6 or B. IL-10 or C. IL-12p70 secretion by un-stimulated or stimulated MoDC or CD1b/c⁺ PBDC. Data represent the means ± SEM of triplicate cultures and is representative of 5-7 separate donors. D. Kinetics of IL-12p70 production by CD1b/c⁺ PBDC matured *in vitro* with GM-CSF or GM-CSF+IL-4 for the indicated times prior to stimulation (24h) with the combination of IL-1β, IFN-γ, CD40L and intact *E.coli*. MoDC were stimulated for 48h with *E.coli*. Data are representative of 4 separate experiments.
Figure 4. Secretion and mRNA expression of soluble factors by immature and mature MoDC and CD1b/c⁺ PBDC.

Immature MoDC (GM-CSF+IL-4) or freshly sorted CD1b/c⁺ PBDC were stimulated for 2 days with either TNF-α,IFN-α,PGE₂ or CD40L or intact *E.coli* and DC examined for mRNA expression by quantitative qRT-PCR. **A.** IL-12p35; **B.** IL-12p40; **C.** IL-23p19 by qRT-PCR as described in the Materials and Methods. **D.** Secretion of IL-18 by un-stimulated or stimulated MoDC or CD1b/c⁺ PBDC. Data are representative of at least 3 separate experiments.
Figure 5. Migratory capacity of immature and mature MoDC and CD1b/c⁺ PBDC.

Immature MoDC (GM-CSF+IL-4) or freshly sorted CD1b/c⁺ PBDC or those stimulated for 8h (A, B) or 24h (C,D) with either TNF-α+IFN-α+PGE₂, or CD40L ± PGE₂ or intact E. coli ± PGE₂ were loaded into the upper transwell chambers and examined for their capacity to migrate toward either medium alone or CCL21 present in the lower transwell chambers. The y-axis shows the number of DC migrating through the transwell membrane (8µm) after 2 hours. Data are representative of 4 separate experiments. E. Secretion of PGE₂ by un-stimulated or stimulated MoDC or CD1b/c⁺ PBDC. Data are representative of 5 separate experiments.
Figure 6. Induction of T cell proliferation by immature and mature MoDC and CD1b/c+ PBDC.

Immature MoDC (GM-CSF+IL-4) or freshly sorted CD1b/c+ PBDC were stimulated for 2 days with the indicated stimuli, washed and used as stimulators (1x10^4) of allo-reactive T cells (1x10^5) in the MLR. On day 5 of the MLR, supernatants were harvested and fresh medium containing 1 μCi ³H-thymidine was added to each well for 8h. Proliferation of T cells stimulated with graded numbers of A. MoDC or B. CD1b/c+ PBDC is shown. Data represent the means ± SEM of triplicate wells. The figure is representative of experiments from 5 separate donors.
Figure 7. Induction of T cell proliferation and IFN-γ secretion by immature and mature MoDC.

Immature MoDC (GM-CSF+IL-4) were stimulated for 2 days with the indicated stimuli and used as stimulators (3x10⁴) of CFSE-labeled CD3⁺ allo-reactive T cells (3x10⁵) in the MLR. On day 5 of the MLR T cells were re-stimulated with the identically conditioned MoDC for 8h in the presence of Brefeldin A and CD4⁺ T cells were assessed for intracellular IFN-γ secretion and proliferation by FACS analysis by gating on CD3⁺CD8⁻ T cells. The figure is representative of experiments from 7 separate donors. Similar profiles were observed when examining CD3⁺ CD8⁺ T cells.
Figure 8. Induction of T cell cytokines secretion stimulation by immature and mature MoDC and CD1b/c+ PBDC.

Immature MoDC (GM-CSF+IL-4) or freshly sorted CD1b/c+ PBDC were stimulated for 2 days with the indicated stimuli, washed and used as stimulators (1x10^4) of allo-reactive T cells (1x10^5) in a MLR. On day 5 of the MLR, supernatants were harvested and T cell cytokine secretion measured by ELISA. A. IL-2 production. B. IL-5 production. C. IFN-γ production. Data are representative of experiments from 5 separate donors.
Figure 9. Peptide presentation and induction of T cell IFN-γ secretion by immature and mature MoDC and CD1b/c⁺ PBDC.

Immature MoDC (GM-CSF+IL-4) or freshly sorted CD1b/c⁺ PBDC were stimulated for 18h with the indicated stimuli, washed, pulsed with the indicated peptides and used as stimulators (1x10⁵) of peptide-specific CTL lines as described in the Materials and Methods section. T cells were assessed for intracellular IFN-γ secretion by flow cytometry. A. NY-ESO-1b₁₅₇-₁₆₅ presentation to NY-ESO-1b-specific CTL line. B. EBV BMLF-1₂₈₀-₂₈₈ presentation to EBV-specific CTL line. Data are presented as the percentage of all CD8⁺ T cells positive for intracellular IFN-γ staining. Data are representative of 3 separate donors.
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Functional comparison of DC generated in vivo with Flt3 ligand or in vitro from blood monocytes: Differential regulation of function by specific classes of physiologic stimuli

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