Characterisation of myeloid receptor expression and interferon alpha/beta production in murine plasmacytoid dendritic cells by flow cytometry

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This is a flow cytometric study of expression of a diverse set of myeloid receptors on murine splenic plasmacytoid dendritic cells (pDCs) and the description of a FACS based assay for measurement of interferon (IFN)α/β. We have extended the known repertoire of PRR expressed on murine pDCs with the novel observation that they express Dectin-2 and contain intracellular MR. In addition, this is the first report of F4/80 and CD200 on murine pDCs. We have confirmed the observation by others that murine pDCs express CD200R, the lectin Dectin-1 and the scavenger receptor CD36. This report also details a flow cytometry-based protocol to measure the production of murine IFNα/β by splenic pDC. Briefly, splenocytes can be stimulated with virus or a TLR9 agonist and IFNα/β production by pDCs is detected following intracellular staining. pDCs are specifically identified by 120G8 staining at 6 h after stimulation with inactivated influenza virus, however the specificity of 120G8 for pDCs is reduced at times later than 12 h. This assay is suitable for use with splenocytes from some mouse strains (129/SvEv), but not others (C57BL/6J), probably due to C57BL6J producing insufficient amounts of IFN following stimulation to be detected by intracellular staining. However, IFN production by C57BL/6J splenocytes is readily detectable by bioassay. In addition to being more sensitive than intracellular staining, the bioassay is also more sensitive than an IFNα ELISA. The comparable sensitivities of these assays are often a critical determinant of the choice of assay and are an important consideration in experimental design.

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1. Introduction

In addition to their plasma cell-like morphology, pDCs express an array of genes associated with lymphocyte development and function (Naik et al., 2005). In contrast, histological studies of the phenotype of human pDCs in inflamed lymph nodes show that pDCs express several markers also expressed on myeloid cells such as macrophages e.g. CD36 and CD68 (Facchetti et al., 1988). Additional evidence suggests a link between myeloid cell and pDC development; mice deficient in IFN consensus sequence binding protein (ICSBP), a transcription factor important for development of myeloid lineage cells, display a loss of pDCs (Naik et al., 2005). There is also a reported association between the accumulation of cells with a pDC phenotype and myelomonocytic leukaemia (Facchetti et al., 2003). Furthermore, macrophage colony stimulating factor can promote pDC development (Onai et al., 2007).

Some clarity has been gained regarding the lineage of pDCs by experiments demonstrating that pDCs can originate from Fms-like tyrosine kinase 3 (Flt3)+ subsets of both common lymphoid progenitors and common myeloid progenitors through a precursor which can also generate conventional DCs (D’Amico and Wu, 2003; Ishikawa et al., 2007; Naik et al., 2007; Onai et al., 2007). Although differences have been observed (Yang et al., 2005), the common lymphoid progenitor and common myeloid progenitor-derived pDCs...
appear indistinguishable by phenotype and function in many studies (Shigematsu et al., 2004; Ishikawa et al., 2007).

In this report we further investigated the myeloid nature of pDCs by flow cytometric analysis of the expression of: the myeloid differentiation antigens F4/80 and 7/4; the lectins mannose receptor, SIGNR1, Dectin-2 and Dectin-1; the scavenger receptors CD36, SR-A and MARCO; and the immunomodulatory receptor-ligand pair CD200R and CD200 (expression described in (Facchetti et al., 1988; Asselin-Paturel et al., 2001; O’Keeffe et al., 2002; Taylor et al., 2002; Hubert et al., 2004; Gavino et al., 2005; Taylor et al., 2005a; Becker et al., 2006)). In addition to being myeloid markers, some are pattern recognition receptors (PRR) and immunomodulatory receptors.

IFNα/β activate a variety of anti-viral immune responses including protecting cells from viral infection and activating DCs, macrophages, T cells and B cells. IFNα/β are therefore critical for viral defence (Muller et al., 1994) and methods to measure type I IFN production are valuable tools in the study of anti-viral immunity. Many cell types can produce type I IFN upon viral infection or stimulation with specific synthetic agonists. pDCs can sense viruses through Toll-like receptor (TLR) and do not require infection to respond to some viruses. Other cells such as macrophages, dendritic cells and fibroblasts produce IFNα/β following viral infection and use cytosolic sensors such as retinoic acid-inducible gene-1 (RIG-1)-like receptors and DNA sensors. Being able to identify which cell produces the IFN is critical for understanding the mechanisms of viral sensing and the relative importance of different cell populations.

There are four main types of assay which can be used to measure IFNα/β production. PCR is commonly used to detect IFNα and IFNβ mRNA expression by pure cell populations such as macrophage or fibroblast cultures. ELISAs and bioassays can be used to measure secreted IFNα/β. Finally, flow cytometry can be used to measure the intracellular accumulation of cytokines (Jung et al., 1993). These assays have different advantages and disadvantages. Detection of mRNA levels by PCR will not identify the IFN producing cell if a mixed population is used and the results may not reflect protein production. However several subtypes of IFNα can be assayed, which is an important consideration due to the differing biological activities of the IFNs and that antibody based approaches may only detect a subset of these. IFNα/β ELISAs and ELISPots allow unambiguous detection of secreted IFNα and IFNβ protein, however again will not identify the IFN producing cell. IFN bioassays are long complex experiments, but detect production of functional IFN proteins using a biologically relevant assay system, protection of cells from viral infection. Intracellular staining for IFNα/β production is a quick method for detecting the production of specific types of IFN protein by specific cell types within a mixed population. Advantages of avoiding purification of pDCs before study include limiting the effects of purification on altering pDC phenotype (Fanning et al., 2006; Grage-Griebenow et al., 2007) and stimulating pDCs in a more natural setting when other cell types are present.

The goals of this study are to investigate expression of a number of myeloid and regulatory receptors on pDC and to describe a method for flow cytometric detection of IFNα/β production. We show that murine pDC express MR, Dectin-1, Dectin-2, F4/80, CD36, CD200 and CD200R and demonstrate intracellular cytokine staining for IFNα/β using inactivated influenza virus as the stimulus for IFN induction.

2. Materials and methods

2.1. Mice

Cells were isolated from 129/SvEv, F4/80 knockout (K/O) (Lin et al., 2005), CD200 K/O (Hoek et al., 2000) or C57BL/6j mouse strains. All animals were at least 6 weeks old and housed and bred at the Sir William Dunn School of Pathology, University of Oxford, according to Home Office and institutional guidelines.

2.2. Spleen cell preparation

Spleens were collected into serum free RPMI (RPMI 1640 (PA A The Cell Culture Company) supplemented with 20 U/ml penicillin, 0.02 mg/ml streptomycin and 2 mM L-glutamine (Sigma)) on ice and single cell suspensions were then prepared by enzymatic digestion. Spleens were digested for 30 min at 37 °C with 1.5 ml per spleen 0.4 mg/ml liberase CI (Roche), 0.1 mg/ml DNase 1 (Roche) in serum free RPMI. Subsequently, cells were forced through a 70 μm cell strainer (Falcon), incubated for 5 min at 37 °C in 5 ml per spleen 0.14 M NH4Cl (Sigma-Aldrich), 17 mM Tris (MP Biomedicals), pH 7.2 in H2O, to lyse red blood cells and then washed three times in serum free RPMI.

2.3. Staining for flow cytometric analysis of receptor expression on pDCs

Fc receptors were blocked in 50 μl FACS wash (0.5% bovine serum albumin (BSA) (Sigma-Aldrich), 5 mM EDTA, 2 mM NaN3 (BDH) in PBS) supplemented with 5% rabbit serum (Invitrogen) and 0.2 mg/ml streptomycin in PBS) supplemented with 5% rabbit serum (Invitrogen) and 4 μg/ml 2.4G2 anti-CD32/CD16 mAb (Unkeless, 1979). In some cases, to show the specificity of 120G8 staining, 100 μ/ml unlabelled 120G8 (Asselin-Paturel et al., 2003) or rat IgG1 isotype control mAb (Serotec) were included in this blocking step. Cells were stained according to standard protocols using the directly labelled or biotinylated primary antibodies listed in Supplementary table A1 (all at 10 μg/ml except anti-B220-PE, which was at 2 μg/ml) and 2 μg/ml of the secondary reagent streptavidin allophycocyanin (SA-APC) (Becton Dickinson) where necessary. Cells were finally fixed for at least 20 min, on ice, in 1% formaldehyde (Sigma) in PBS. For intracellular staining, cells were first fixed in 1% formaldehyde and then 0.2% saponin (Sigma-Aldrich) was included in all blocking, staining and washing steps.

2.4. Dil-AcLDL association assay

2.7 x 10^7 129ICR spleen cells/ml were pre-incubated in a volume of 0.75 ml serum free RPMI alone or supplemented with 50 μg/ml polyinosinic acid (poly I) (Sigma-Aldrich) or 50 μg/ml polycytidylic acid (poly C) (Sigma-Aldrich) for 30 min at 37 °C. 2.5 μg/ml 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-labelled acetylated low-density lipoprotein (Dil-AcLDL) (Invitrogen) (Dil-
AcLDL) was then added for 1 h at 37 °C. Cells were subsequently stained with 120G8 to identify pDCs.

2.5. Spleen cell stimulation

2 × 10^7 spleen cells were stimulated with 400 HAU non-infectious β-propiolactone inactivated influenza virus, (A/Guangdong/25/93) (H3 N2) that was grown in embryonated hens eggs and subsequently purified by Dr. M Hocart (CSL Limited), or with 1 µM of the TLR9 agonist CpG ODN 2216 (Invivogen) for 6–23 h in 2 ml or 0.75 ml volumes. Supernatants were collected and stored at −20 °C until analysis for IFNα content by ELISA or bioassay. To measure IFNα/β production by intracellular staining 1 µl GolgiPlug (Becton Dickinson) was added per ml of media after 5 h and the incubation continued. At the end of the stimulation, cells were detached from the plate by scraping, washed 3 times in PBS and then fixed in 1% formaldehyde before staining.

2.6. Intracellular staining for murine IFNα and IFNβ

2.6.1. Standard intracellular staining protocol for detection of IFNα or IFNβ

Following stimulation, the fixed cells were blocked in 50 µl permeabilizing FACS wash containing 0.2% saponin, 5% rabbit serum or sheep pAb against IFNα or IFNβ and then stained with 100 µl of 2 µg/ml 120G8-biotin or rat IgG1-biotin, or 120G8-Alexa647 and 4EA1-biotin for 1 h on ice. Finally, cells were washed three times in 50 µl FACS wash, stained with 100 µl of 2 µg/ml SA-APC for 30 min on ice, washed three times again with 50 µl FACS wash and then fixed in 1% formaldehyde.

2.6.2. Staining with rabbit pAb against IFNα or sheep pAb against IFNα/β

Cells were blocked in 50 µl permeabilizing FACS wash supplemented with 0.2% saponin, 4 µg/ml 2.4G2 and 5% goat serum for 30 min on ice and then stained with 100 µl 10 µg/ml 120G8-biotin or rat IgG1-biotin and 200 U/ml rabbit pAb against mouse IFNα or 1/1000 dilution sheep pAb against IFNα/β diluted in the blocking solution for 1 h on ice. Cells were then washed three times with 50 µl FACS wash, incubated for 30 min on ice with 100 µl 2 µg/ml SA-APC diluted in FACS wash, washed three times in 50 µl FACS wash and then fixed in 1% formaldehyde.

2.6.3. Staining with RMMA-1 (anti-IFNα mAb) or co-staining for IFNα and IFNβ

Cells were blocked in 50 µl permeabilizing FACS wash including 0.2% saponin, 5% rabbit serum and 5% goat serum for 30 min on ice and then stained with 100 µl 2.5 µg/ml RMMA-1 (rat mAb against IFNα) or 10 µg/ml 7FD3 mAb diluted in the blocking solution for 1 h on ice. Cells were then washed three times in 50 µl FACS wash and incubated with 100 µl of 10 µg/ml goat anti-rat IgG-AlexaFluor488 for 30 min on ice. Subsequently, cells were washed three times and re-blocked in 50 µl FACS wash containing 0.2% saponin and 10% rat serum for 30 min, on ice, before staining with 100 µl 10 µg/ml 120G8-biotin or rat IgG1-biotin, or 120G8-Alexa647 and 4EA1-biotin for 1 h on ice. Finally, cells were washed three times in 50 µl FACS wash, stained with 100 µl of 2 µg/ml SA-APC for 30 min on ice, washed three times again with 50 µl FACS wash and then fixed in 1% formaldehyde.

2.7. FACS analysis

Data were acquired on the FACS Calibur system (BD) and analysed using FlowJo software (Tree Star Incorporated). To analyse the expression of proteins by pDCs, debris was gated out by forward scatter and side scatter characteristics and 120G8+ cells were selected by comparison to control staining. To analyse the phenotype of pDCs, the percentage of cells within each quadrant on dot-plots is indicated.

2.8. IFNα ELISA

The IFNα ELISA was adapted from a protocol by Dr. S. Cartland (Yrlid et al., 2006). Briefly, 96 well ELISA plates (Corning Incorporated) were coated overnight at 4 °C with 50 µl/well of 4 µg/ml 4E1 mAb. The next day the wells were washed in wash solution (0.05% Tween (Sigma-Aldrich) in PBS) and then incubated with 200 µl/well ELISA blocking solution (10% heat inactivated foetal calf serum in PBS) for 2 h at 37 °C. The blocking solution was then removed and 35 µl of sample or murine IFNα standard (PBL Biomedical Laboratories), serially 2-fold diluted in duplicate (from 40,000 U/ml in the case of the standard) were incubated on the plate for 2 h at room temperature. The plate was then washed with wash solution and then incubated for 1 h at room temperature with 50 µl/well 100 U/ml rabbit pAb against IFNα diluted in blocking solution. Wells were washed with PBS and incubated for 1 h at room temperature with 50 µl/well alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma-Aldrich) diluted 1/1000 in PBS. Subsequently wells were washed with PBS and then alkaline buffer (50 mM Na2CO3 (Fischer Scientific), 1 mM MgCl2, 6H2O (BDH) pH9.8 in H2O) and 1 mg/ml p-nitrophenyl phosphate (Sigma-Aldrich) in alkaline buffer was added. The plate was incubated at room temperature and, when a strong colour was visible, absorbance at 405 nm was recorded on an Anthos htl spectrofluorometer. To estimate the amount of IFNα in the samples, the mean absorbance for duplicate samples was compared to a standard curve of the IFNα standard. The limit of detection of IFNα was estimated from the standard curve as the lowest amount of IFNα that gave a detectable signal (A405 nm).

2.9. IFN bioassay

This assay measured IFN by the ability to inhibit Cocal virus induced cytopathic effect (CPE) on L929 cells. Monolayers of L929 cells in 96 well tissue culture plates were incubated with duplicate 2-fold serial dilutions from 250 U/ml of either IFNα or samples diluted in serum free RPMI overnight at 37 °C. Subsequently cells were challenged with 250–2150 pfu/ml Cocal virus (obtained from Prof. W. James, University of Oxford, UK) diluted in serum free RPMI. As
controls, cells were incubated with serum free RPMI alone (cell control) or without IFN, but with Coval virus only (virus control). After 2 days the point of 50% CPE was determined microscopically and the amount of IFN in the samples was estimated by comparison with the standard curve from the IFNαA. The amount of IFNαA which gave 50% CPE was determined to estimate the sensitivities of the bioassay. Below this point IFNαA was rarely detectable.

3. Results

3.1. Identification of pDCs

Murine pDCs are defined as B220+CD11c+CD11b−Ly6C+ cells (Liu, 2005). More recently developed mAb, such as 120G8, anti-Siglec-H mAb and anti-PDCA-1 mAb, have simplified the study of pDCs and allow identification of this population by use of a single mAb. The mAb 120G8 was used to define murine spleen pDCs in this study. To confirm that 120G8 identified pDCs, spleen cells were co-stained with 120G8 and for B220, CD11c, CD11b or Gr-1 (Ly6C/G). 120G8 stained B220+CD11c+CD11b−Gr-1−/low cells, matching the published phenotype of pDCs (Fig. 1A). Fig. 1B shows staining of splenocytes with Alexa488 labelled 120G8 when rat IgG1 isotype control mAb was included in the blocking step compared to control staining where an excess of unlabelled 120G8 was included in the blocking step during staining. A gate highlighting the 120G8+ cells is indicated and is an example of that used to define pDCs in subsequent experiments. 120G8+ cells also expressed Siglec-H, another marker of pDCs (Fig. 1C).

3.2. pDCs express myeloid restricted antigens

Spleen pDCs from the 129/SvEv mouse strain were analysed by flow cytometry for expression of the myeloid differentiation antigens F4/80 and 7/4, the lectins Dectin-1, Dectin-2, mannose receptor and SIGNR1, the scavenger receptors SR-A, MARCO and CD36, and the immunomodulatory receptor CD200R and its ligand CD200. When possible (as shown), antibody specificity was confirmed by staining cells deficient in the respective receptors. pDCs were defined as 120G8+ cells, as described in Fig. 1B.

The myeloid differentiation antigen F4/80 was detected on the surface of 129/SvEv pDCs (Fig. 2A). The staining was specific as F4/80 was not detected on F4/80 K/O pDCs, but was expressed on the corresponding control C57BL/6 J pDCs. The 7/4 antibody, which recognizes an unknown antigen expressed on myeloid cells, also clearly stained 129/SvEv pDCs (data not shown). However, 7/4 staining was also present on Balb/c pDCs, which do not express the 7/4 antigen (Hirsch and Gordon, 1983), showing that this antibody was binding pDCs non-specifically (data not shown).

pDCs also expressed low, but reproducibly detectable, levels of Dectin-1 (Fig. 2B) and Dectin-2 (Fig. 2C) on their surface. Mannose receptor expression was detected in permeabilized
Fig. 2. Expression of myeloid restricted antigens on pDCs. Single-cell suspensions of spleen cells (from the 129/SvEv strain unless otherwise stated) were stained with 120G8 and with mAb against (A) F4/80 \( (n=3 \text{ on 129/SvEv spleen cells; } n=2 \text{ on C57BL/6J and F4/80 K/O spleen cells}) \), (B) Dectin-1 \( (n=3) \), (C) Dectin-2 \( (n=3) \), (D) mannose receptor \( (n=2) \), (F) CD36 \( (n=3) \), (G) CD200R \( (n=3) \), (H and I) CD200 \( (n=3) \) or with an appropriate isotype control mAb. Black histograms show receptor expression on pDCs compared to isotype control staining (grey filled histogram). In (D) spleen cells were permeabilized with saponin during staining. In (I) C57BL/6J and CD200 K/O spleen cells were also stained for CD200 expression \( (n=3) \). In (E) scavenger receptors were detected on pDCs by pre-incubating 129ICR spleen cells in media alone (no ligand) or in media supplemented with 50 mg/ml poly I (SR block) or 50 µg/ml poly C (control) for 30 min prior to addition of 2.5 µg/ml Dil-AcLDL (a SR ligand). Cells were subsequently stained with 120G8 to identify pDCs and the graph shows Dil-AcLDL association (geometric mean fluorescence) with pDCs \( (n=1) \).
pDCs, but not on the surface of pDCs (Fig. 2D and data not shown). Mannose receptor expression could not be detected on the surface or within permeabilized C57BL/6J pDCs, therefore, as the mannose receptor K/O is on the C57BL/6J background, the specificity of mannose receptor staining could not be confirmed (data not shown). Detection of SigIR on the surface of pDCs from the 129/SvEv strain varied from negative to intermediate levels in three experiments, and SigIR could not be detected on pDCs from C57BL/6 × 129 (the positive control strain for the K/O) or SigIR KO mice (data not shown). Murine spleen pDCs therefore expressed several lectins with PRR functions.

Scavenger receptors are endocytic receptors expressed on macrophages which facilitate the recognition and uptake of endogenous and exogenous ligands. To investigate whether pDCs expressed scavenger receptor activity, spleen cells were incubated with the fluorescently labelled SR ligand DiI-AcLDL and then stained with 120G8 to identify pDCs. pDCs were found to associate with DiI-AcLDL (Fig. 2E). To test whether the association of DiI-AcLDL with pDCs was specific, spleen cells were incubated with the scavenger receptor ligand polyI or the control ligand polyC, before and during addition of DiI-AcLDL. PolyI reduced the association of DiI-AcLDL with pDCs, while polyC did not (Fig. 2E), indicating the expression of a functional scavenger receptor on pDCs. pDCs also expressed the scavenger receptor CD36 (Fig. 2F). There was no evidence that pDC expressed SR-A: the anti-SR-A mAb 2F8 did not reduce DiI-AcLDL association with pDCs; the level of DiI-AcLDL association with SR-A K/O pDCs was similar to that with control 129ICR pDCs; and staining of pDCs with the anti-SR-A 2F8 mAb was also detected on SR-A K/O pDCs (data not shown). In addition, the detection of MARCO, a receptor with regulated expression on macrophages, on pDCs was highly variable (data not shown).

CD200R and CD200 are an immunomodulatory receptor–ligand pair which inhibit myeloid cell functions. pDCs expressed readily detectable CD200R and CD200 (Fig. 2G and H). The detection of CD200 was specific as no staining for CD200 was detected on CD200 K/O pDCs, while CD200 was clearly detectable on C57BL/6J pDCs (Fig. 2I). In summary, a range of myeloid antigens including receptors with pattern recognition or immunomodulatory functions were detected on murine spleen pDCs.

The phenotype of pDC from mouse blood was analysed as a comparison to the spleen pDC population. pDCs from blood had a different profile of receptor expression compared to spleen pDCs. Only 7/4 and mannose receptor were detected on blood pDCs, while no F4/80 or Dectin-1 expression was observed (data not shown). Detection of MARCO and Dectin-2 expression was variable (data not shown).

3.3. Identification of mAb for measuring IFNα/β production by murine splenic pDCs

The protocol developed here for the stimulation of spleen cells and measurement of IFNα/β production by pDCs was based on the conditions for stimulation of spleen cells with inactivated influenza virus known to induce IFN production measurable by a bioassay (Miller and Anders, 2003). Initial experiments used spleen cells from the 129/SvEv mouse

Fig. 3. FACS comparison of intracellular cytokine staining with different anti-IFNα/β antibodies. 1 × 10^7 spleen cells/ml were incubated for 9 h (or 6 h for staining with 4EA1) in media alone or with 200 HAU/ml inactivated influenza virus in a volume of 2 ml per treatment. After 5 h GolgiPlug was added and the incubation continued. Cells were fixed in 1% formaldehyde, permeabilized with saponin, stained with 120G8 to identify pDCs and several antibodies against murine IFNα/β were tested for their ability to detect IFNα or IFNβ production. The following antibodies were tested: an anti-IFNα pAb; RMMA-1 and 4EA1, two rat mAb against IFNα; an anti-IFNα/β pAb; and 7FD3, a rat mAb against IFNβ. The grid lines were set by control staining for 120G8 and for staining with the anti-IFNα/β antibodies following incubation with media alone and applied to the corresponding graph following stimulation with inactivated influenza virus. The numbers on each graph show the percentage of total cells within each quadrant.
Fig. 4. Identification of pDCs as the IFNα and IFNβ producing cells following stimulation with inactivated influenza virus. (A) Spleen cells were stimulated with media alone or 1 µM CpG ODN2216 for 9 h (n = 2). In (B) and (C) spleen cells were incubated for 6 h with inactivated influenza virus or media alone. In (B) cells were permeabilized with saponin and stained with 120G8 and with the mAb 4EA1 and 7FD3 to measure IFNα and IFNβ production (n = 1). In (C) cells were stained with 120G8 and for CD11c, B220 or CD11b expression (n = 2).
strain as others have shown this strain has a higher number of pDCs in the spleen and produced more IFNα following stimulation compared to other strains (Asselin-Paturel et al., 2003). Single-cell suspensions of spleen cells were prepared by digestion with liberase and only preparations where there was greater than 90% cell viability, as assessed by trypan blue staining (data not shown), were used in subsequent experiments. GolgiPlug (brefeldin A) was added during the stimulation (after 5 h) to inhibit secretion and promote retention of IFNα/β within the pDCs. The typical staining protocol for the measurement of intracellular antigens by flow cytometry employed was to fix cells, permeabilize them with saponin and stain with anti-pDC and anti-IFNα/β Ab. Various factors were tested within this general protocol. Initially several anti-IFNα/β Ab were tested for their ability to detect IFNα and IFNβ. Fig. 3 shows that a rabbit pAb against IFNα, RMMA-1 mAb, 4EA1 mAb and the mAb 7FD3, can be used to measure IFNα and IFNβ production by pDCs (120G8+ cells). In addition 4EA1 and 7FD3 could also measure IFNα and IFNβ production by pDCs when splenocytes were stimulated with the defined TLR9 agonist CpG ODN 2216 (Fig. 4A). Approximately half the pDCs expressed IFNα 6 h after stimulation (Fig. 3 and data not shown). A similar proportion expressed IFNβ following the same stimulation. Triple staining with 120G8, 4EA1 and 7FD3 showed that the majority of pDCs producing IFNα were also IFNβ+ and IFNα− cells were IFNβ− (Fig. 4B). As the expression of the antigen recognized by 120G8 is upregulated on non-pDCs by type I IFN (Asselin-Paturel et al., 2003; Blasius et al., 2006), the specificity of 120G8 for pDCs following stimulation was confirmed. Following stimulation of spleen cells for 6 h with influenza virus or media alone, 120G8+ cells were CD11c+ B220− and CD11b−, corresponding to pDCs (Fig. 4C).

3.4. Length of stimulation affects detection of pDC by 120G8

Use of only one mAb to identify pDCs enables the measurement of multiple variables within one sample. However, as the expression of 120G8 is reported to be upregulated on non-pDCs by IFNα, we examined the ability to detect IFNα producing pDCs over a time course of 23 h. Fig. 5 shows that following stimulation with inactivated influenza virus the ability to detect a clear population of pDCs diminishes after approximately 12 h. Nonetheless, following 6 h stimulation with inactivated influenza virus 120G8 stains a population of CD11c+ B220− CD11b− cells, matching the phenotype of pDCs, and is therefore specific under these conditions (Fig. 4C). At later time points a combination of mAb may be required to identify pDCs clearly.

3.5. Intracellular staining detects IFNα/β production by 129/SvEv but not C57BL/6J pDC

In the IFNα/β induction experiments described so far, spleen cells were isolated from 129/SvEv mice and IFNα/β production, following inactivated influenza virus or CpG ODN stimulation, was readily detectable by intracellular staining (Figs. 3 and 4). However, in contrast, this intracellular staining protocol failed to reproducibly measure IFNα production by C57BL/6J spleen cells stimulated with inactivated influenza virus, although multiple time points and virus doses were tested (data not shown). As others report that the disparity of IFN production levels between these two mouse strains may be in part due to C57BL6J spleens containing fewer pDCs (Asselin-Paturel et al., 2003), spleen cells were stained with 120G8 and the percent pDCs was calculated. The 129/SvEv and C57BL/6J splenocyte preparations had a similar percent
of pDCs of total cells; 1.1 ± 0.3% in 129/SvEv and 1.1 ± 0.1% in C57BL/6J splenocytes. This suggests that C57BL/6J pDCs spleen cells have a lower IFN inducing capacity compared to 129/SvEv spleen cells.

3.6. Intracellular staining is less sensitive compared to an IFNα ELISA and bioassay

To compare the levels of IFN produced by pDC from different strains of mouse, 129/SvEv and C57BL/6J murine splenocytes were stimulated with inactivated influenza virus or CpG ODN2216 for 6–23 h and IFN production was measured by bioassay. As measured by bioassay, C57BL/6J spleen cells produced less IFN than 129/SvEv spleen cells over a time course of 6–23 h (Fig. 6). The lower levels of IFN produced by C57BL/6J splenocytes could not detect IFNα production by C57BL/6J splenocytes while the bioassay could (data not shown and Fig. 6), show that the bioassay is more sensitive than the intracellular staining protocol.

ELISA based assays are also commonly used to measure IFN secretion by cells. The IFNα ELISA could detect IFNα production by 129/SvEv spleen cells stimulated with inactivated influenza virus and CpG ODN 2216 (data not shown), which was also detected by intracellular staining. However, the IFNα ELISA did not consistently detect IFNα production by C57BL/6J spleen cells stimulated with inactivated influenza virus (data not shown). The lower limit of detection in an IFNα ELISA assay (for an IFNα standard) was approximately 800 U/ml (Fig. 7). The comparative sensitivity of the ELISA to the bioassay was investigated by comparing the lowest level of IFNα detected by each assay. The bioassay could clearly detect lower level of IFNα than the ELISA (Fig. 7).

4. Discussion

The expression of a range of myeloid associated markers by pDCs is interesting given the lack of clarity over the exact lineage of pDCs, the flexibility recent evidence suggests exits in pDC development as well as the rapidly emerging data on novel PRR. Confusion over the lineage of pDCs may have arisen because pDCs share characteristics of both lymphoid and myeloid cells while lacking some classical markers of both lineages. This study therefore highlights that pDCs do express a range of myeloid antigens (the expression of lymphoid associated antigens was not studied). We show that pDCs express the lectins mannose receptor (in a strain dependent manner), Dectin-1 (at low levels) and Dectin-2. pDCs have been described to express the scavenger receptor CD36 and consistent with this exhibited scavenger receptor function by associating with the classic scavenger receptor ligand acLDL. In addition pDCs expressed the macrophage marker F4/80 and the immunomodulatory receptor CD200R together with its ligand CD200. In this respect pDCs resemble macrophages and myeloid DCs. By contrast, pDCs also express lymphoid genes and therefore, while the function of many molecules expressed by pDCs is unknown, their phenotype clearly appears diverse.

As many of the receptors studied here are PRR this expands the repertoire of potential pathogen sensing mechanisms on pDCs. Some of the proteins, such as F4/80, 7/4 and Dectin-2 have no clear known function. Others are expressed by myeloid cells and have roles in non-viral pathogen recognition, e.g. Dectin-1. In addition, the mannose receptor, DC-SIGN family members and some scavenger receptors recognize viruses as well as non-viral pathogens. In a recent microarray study of the gene expression profiles of human and mouse lymph node resident DCs (conventional DCs and

![Fig. 6. Bioassay of IFN production by 129/SvEv and C57BL/6J spleen cells.](image1)

![Fig. 7. Comparison of sensitivity of IFNα ELISA and bioassay.](image2)
pDCs), the gene expression profiles of conventional DCs and pDCs were found to cluster together, with limited overlap with lymphoid and myeloid gene expression (Robbins et al., 2008). The expression of these myeloid receptors by pDCs may reflect their ontogeny and influence the plasticity of their functional properties as they differentiate. Current studies are underway to assess whether any of these receptors play a functional role in type I IFN induction in these cells.

Murine splenic pDCs express both partners of the immunomodulatory receptor–ligand pair CD200R and CD200. Currently the significance of the co-expression of both receptor and ligand within the same cell population is unknown. The detection of CD200R on pDCs is supported by data showing binding of a CD200-Ig fusion protein to mouse spleen pDCs (Fallarino et al., 2004; Fallarino et al., 2005). Although there have been no previous reports of CD200 expression on mouse pDCs, rat pDCs clearly express CD200 (Hubert et al., 2004). It is interesting that unstimulated pDCs express both CD200 and CD200R, while in macrophages CD200 expression is inducible following stimulation and CD200R is constitutively expressed and downregulated following stimulation (Dr. S. Mukhopadayay, personal communication). This suggests that the expression of CD200 and CD200R in pDC and macrophages is differentially regulated. By analysing spleen cells from mice deficient in MyD88 or IFNα/βR it was found that the expression of CD200 and CD200R on freshly isolated spleen pDCs does not depend on either of these molecules (data not shown). This is in contrast to findings in macrophages, were CD200 expression is MyD88-dependent.

This study to identify receptors expressed on murine pDCs illustrates several important technical constraints. A strain difference was found between pDCs from 129/SvEv mice and the C57BL/6 mouse strains regarding mannose receptor expression, and similarly between 129/SvEv and C57BL/6 × 129 pDCs for SIGNR1 expression. Levels of IFN production also differed between 129/SvEv and C57BL/6 spleenocytes, illustrating functional as well as phenotypic differences. Furthermore, the detection of MARCO and SIGNR1 expression was variable, possibly reflecting the idea that C57BL/6J pDCs have a lower intrinsic type I IFN producing capacity following viral stimulation than 129/SvEv pDCs, perhaps due to polymorphisms in the induction pathway.

Flow cytometry-based intracellular cytokine assays have now been developed to detect IFNα production by murine, human and porcine cells. For example, intracellular assays can detect IFNα production by murine bone marrow-derived DCs stimulated with poly(I:C) (Diebold et al., 2003) and by bone marrow-derived Flt3L pDCs stimulated with inactivated influenza virus (Diebold et al., 2004). Similarly, intracellular staining can detect IFNα/β production by spleen pDCs from mice infected with MCMV or stimulated with CpG ODN (Sjolin et al., 2006). These techniques, and others (Asselin-Paturel et al., 2005; Chan et al., 2006), to detect murine IFNα/β, used the monoclonal antibodies RMMA-1 (PBL Laboratories), F18 (Hycult labs) or RMMB-1 (PBL Laboratories) for cytokine staining. Here we describe a method for stimulation of spleenocytes with a virus (inactivated influenza) or TL9 agonist (CpG ODN 2216) and measurement of IFNα/β production by pDCs by intracellular staining using the 120G8 mAb to identify pDCs and several anti-IFNα/β Ab (RMMA-1, 4EA1, 7FD3, polyclonal anti-IFNα). This assay allows for measurement of protein production, identification of whether IFNα or IFNβ is produced, and identification of the IFNα producing cell population.

The surface phenotype and expression of markers by immune cells often change following stimulation. 120G8 specifically identifies non-stimulated spleen pDCs by flow cytometry (Asselin-Paturel et al., 2003). Treatment with IFN, or stimuli that induce IFN production, upregulates expression of the protein recognized by 120G8, BST2, on many other cells such as B, DC, T, NK and NK T cells (Asselin-Paturel et al., 2003; Blasius et al., 2006). These experiments were conducted following longer stimulation times (20–60 h) than typically used here. The 120G8 mAb is specific for pDCs following 6 h stimulation with inactivated influenza virus, but following longer stimulation times (e.g. 12 h) the antigen recognized by the 120G8 mAb is upregulated on other spleen cells.

Spleen cells from different mouse strains produced different levels of type I IFN following stimulation with inactivated influenza virus. For example, while intracellular staining could readily detect IFNα/β production by 129/SvEv pDCs, no IFNα/β production by C57BL/6J spleen cells was reproducibly detected by this method. In addition, the time course study of IFN secretion by spleen cells stimulated with inactivated influenza virus or CpG ODN showed that C57BL/6J spleen cells produced less IFN than 129/SvEv spleen cells. It has been reported that 129/Sv spleens have a higher proportion of pDCs compared to total cells than C57BL/6J (Asselin-Paturel et al., 2003). Interestingly, the study by Asselin-Paturel et al. using influenza virus stimulation of equal numbers of purified pDCs also showed that 129/Sv pDCs produced more IFNα than C57BL/6 pDCs on a per cell basis (Asselin-Paturel et al., 2003). We show that the percentage of 120G8+ spleen cells was not significantly different between the 129/SvEv and C57BL/6J strains. However, the different level of IFN production by 129/SvEv and C57BL/6J spleenocytes measured by the bioassay and the inability of the intracellular staining protocol to detect IFNα/β production by C57BL/6J spleen pDCs stimulated with inactivated influenza virus seen here supports the idea that C57BL/6J pDCs have a lower intrinsic type I IFN producing capacity following viral stimulation than 129/SvEv pDCs, perhaps due to polymorphisms in the induction pathway.

5. Conclusion

The three different assays compared here each have strengths and weaknesses that must be considered when choosing between them. The comparative sensitivity of the intracellular staining protocol, ELISA and bioassay was often a critical factor determining which assay could be used. The bioassay is clearly the most sensitive assay as it can detect IFN production when the other assays fail to do so, such as following inactivated influenza virus or CpG ODN stimulation of C57BL/6J spleen cells. However, both the intracellular staining assay and ELISA can be used to measure more robust IFNα/β production, such as by 129/SvEv spleenocytes. In addition, while the FACS based assay is preferable for measurement of type I IFN production during relatively short incubation times (6–9 h), the IFNα ELISA and bioassays described here are not
complicated by the detection of the pDCs, and therefore can measure secreted IFNβ over longer incubation periods. An important feature of the intracellular cytokine staining assay described here is the ability to define the IFN producing population. In addition, the FACS based assay detailed here can distinguish between IFNα and IFNββ production, while the bioassay can detect both type I or type II IFN, and specific blocking antibodies must be used in a more complicated experimental set up to define the type of IFN. A biological assay for IFN production does, however, have the advantages of measuring functional IFN secretion, which is protective against viral infection. The intracellular IFNαβ assay described in this study is therefore an assay to measure IFNαββ production by pDCs when stimulated within a mixed cell population and is a useful tool to dissect anti-viral immune responses.

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Appendix A. Supplementary data


References


