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Identification of a lineage negative cell population in bovine peripheral blood with the ability to mount a strong type I interferon response

Amanda Gibson [a,1], Suhel Miah [b], Philip Griebel [c], Joe Brownlie [a], Dirk Werling [a,*]

[a] The Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Hertfordshire AL9 7TA, UK
[b] Institute of Orthopaedics and Musculoskeletal Science (IOMS), University College London (UCL), Royal National Orthopaedic Hospital (RNOH) NHS Trust Campus, Brockley Hill, Stanmore, Middlesex HA7 4LP, UK
[c] Vaccine and Infectious Diseases Organisation/International Vaccine Centre (VIDO/Intervac), School of Public Health, University of Saskatchewan, 120 Veterinary Road, Saskatoon, SK, Canada S7N 5E3

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ABSTRACT

Lineage negative dendritic cells, or natural interferon-producing cells (NIPC), also referred to as plasmacytoid dendritic cells (pDC) constitute a small population of leukocytes secreting high levels of type I interferon (IFNα/β) in response to certain danger signals. Here, we provide initial data towards the identification of so far uncharacterised circulating bovine pDC like cells. A lineage negative cell population (LIN^- cells) was isolated from PBMC which showed characteristics similar to that of pDC in other species. Isolated LIN^- cells presented lymphoid morphology with a semi-crescent nucleus, extensive ER and Golgi network; indicative of pDC. In addition phenotypic analysis of LIN^- cells described them as distinct from other bovine DC subsets; expressing both lymphoid and myeloid surface markers. LIN^- cells did not express lineage specific markers, but were MHC class II^+, $CD45RO^+$, $CD80/86^+$, $CD6^+$, $WC1^+$, $CD26^+$ and expressed the myeloid markers CD205, CD172a and CD11a. In keeping with pDC, LIN^- cells express TLR7 mRNA transcripts; however, in a resting state do not express TLR8 or TLR9. Functionally, LIN^- cells, but not PBMC, monocytes and monocyte derived DC produce large amounts of IFNα/β in response to different CpG oligonucleotides. Taken together, we present data suggesting that an enriched circulating population of bovine LIN^- cells are uniquely capable of producing IFNα/β in response to CpG oligonucleotides and thus this population likely contain the functional equivalent of bovine pDC.

1. Introduction

Plasmacytoid DCs (pDCs) are thought to constitute ~1% of PBMCs in humans, are largely lineage negative and accumulate at sites of inflammation such as allergic mucosa (McKenna et al., 2005). Early work described a cell type capable of producing large amounts of type I interferon (IFNα/β) in response to both infectious and inactivated virus inducing an anti viral state (Seeds et al., 2006) via activation of PKR, 2′-5′ OAS, Mx protein and cytokine production. This process culminates in viral nucleic acid degradation, protein synthesis arrest, and cell death (Haller and Kochs, 2002; Hovanessian, 2007). IFNα/β also have direct affects on other immune cells by increasing MHC class I expression (Keir et al., 2002), inducing maturation of circulating dendritic cells (cDC) (Honda et al., 2003; Martinez et al., 2008) activating both natural killer (NK) cells (Martinez et al., 2008) and $CD8^+$ memory T cell function (Montoya et al., 2002). Although pDCs are clearly not the only cell able to produce IFNα/β, they are unique in their ability to produce large amounts of IFNα/β – a virtue owed to coupling of innate cellular receptors such as TLR7 and TLR9 with rapid IFNα/β synthesis (Diebold et al., 2004).

Although human, murine and to a lesser extent rat pDC are well characterised both phenotypically and functionally, the knowledge of pDC in other species, in particular domestic animals, is less advanced. Porcine pDC have been described phenotypically, as well as based on their unique ability to induce potent IFNα/β secretion upon viral stimulation. Distinct from cDC, blood resident porcine pDC are typically $CD172a^+$, $CD4^+$, $CD8^{low}$, MHC class II^{low}, $CD80/86^{low}$ and $CD45RA^{low}$ and require IL-3 or viral activation as survival factors in culture (Summerfield et al., 2003). Viral replication is not necessary for recognition by porcine pDC, as UV-inactivated transmissible gastroenteritis virus (TGEV) also induced IFNα/β production in the above study. Similar pDC populations have also been described within porcine secondary lymphoid organs and the spleen, displaying a $CD172a^+$, $CD4^+$, $CD1^{low}$ and $CD80/86^{low}$ phenotype. Steady state cytokine production of pDC observed in both blood and spleen fractions

* Corresponding author. Address: Department of Pathology and Infectious Diseases, Royal Veterinary College, Hawkshead Lane, Hatfield, Hertfordshire AL9 7TA, UK. Tel.: +44 (0)1707 666 358; fax: +44 (0)1707 661 464.
E-mail address: dwerling@rvc.ac.uk (D. Werling).
[1] Present address: Health Protection Agency, National Mycobacterium Reference Laboratory (NMRL), Abernethy Building, 2 Newark Street, E1 2AT, UK.

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showed a tendency for a higher production of IFNα, TNFα, IL-12 and IL-10 than blood pDC (Jamin et al., 2006). Upon stimulation, porcine pDC have been shown to mature, down-regulate endocytic antigen uptake and increase expression of both MHC class II and co-stimulatory molecules. Furthermore, supernatants from matured pDC induced the maturation of monocyte derived dendritic cells (moDC), subsequently enabling T cell proliferation (Guzylack-Piriou et al., 2004; Summerfield et al., 2003).

Very recently, pDC were identified in the B cell depleted $CD45RB^{+}/CD11c^{-}$ fraction of low density cells within the afferent lymph and blood of sheep. In line with porcine pDC, ovine equivalents are MHC class II^{low}, $CD80/86^{low}$ and $CD45RA^{low}$ and respond to viral stimulation by producing IFNα/β (Pascale et al., 2008). Additionally, ovine pDC were found to be $CD4^{low}$ and $CD62L^{+}$, with little variation observed between lymph and blood populations. IFNα/β production was restricted to these pDC in response to CpG ODN, TGEV and influenza virus. These cells were subsequently found to express high levels of TLR7, TLR9 and like human pDC, IRF7 at the mRNA level. Although ovine pDC increased expression of MHC class II molecules and CD86 upon stimulation, the ability to induce $CD4^{+}$ T cell proliferation and IFNγ production was far greater in $CD11c^{+}$ DC (Pascale et al., 2008). However, the capacity for pDC to induce maturation of DC remains to be observed in ovine cultures. Interestingly, pDC injected intradermally were found to be located within the draining lymph in the above study, indicating a migratory role for these cells.

Ruminant cells have also been described to respond to Type A CpG-ODN with the production of IFNα/β, showing a species-specific pattern (Mena et al., 2003), however the exact cell population responding to the synthetic DNA was not established in this study. Interestingly, a population of IFNα/β producing cells were identified in lymph nodes of cattle acutely infected with Bovine Viral Diarrhoea Virus (BVDV) (Brackenbury et al., 2005). BVDV, classified in the same viral family as Hepatitis C (Meyers and Thiel, 1996), is known to interfere with IFNα/β production, which seems to contribute to the development of persistently infected calves (Baigent et al., 2004; Baigent et al., 2002). This IFNα/β producing cell population was found to be not infected with BVDV, but resident within the T cell region of the paracortex (Brackenbury et al., 2005). Isolation of these cells from lymph node preparations revealed a cell population with the phenotype $CD14^{+}$, $CD11b^{+}$ and $CD172a^{+}$, but $CD4^{-}$ and $CD45RB^{-}$ (Brackenbury et al., 2005). This phenotype would normally be attributed to cells of myeloid origin, and thus may not represent the bovine equivalent of human pDC. However, expression of CD62L, along with the detection of this enriched population within the T cell area implies at least a functional resemblance to human pDC (Brackenbury et al., 2005).

Given our lack of knowledge regarding the pDC equivalent in ruminants, the main aim of the present study was to characterise a bovine equivalent to pDCs described in other species through surface antigen expression and subsequent functional analysis. Here we describe a circulatory lineage negative (LIN^{-}) cell population isolated from PBMC, expressing both myeloid and lymphoid surface markers and sharing pDC attributed morphology. We show that LIN^{-} cells are uniquely capable of producing IFNα/β in response to CpG ODN (Type A and Type B) in comparison to monocytes and moDC.

2. Materials and methods

2.1. Animals and blood collection

Whole blood was collected from healthy Friesian–Holstein bullocks into 10% acid citrate dextrose buffer as anti-coagulant in accordance with Home Office regulations. All animals were tested for the absence of BVDV antibodies and viral RNA by the Veterinary Laboratories Agency, Weybridge, UK.

2.2. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cell (PBMC) isolation was performed as previously described after recovering the buffy coat interface layer (Werling et al., 1999; Werling et al., 2004). Briefly, PBMC were collected after density centrifugation of the buffy coat at 1200 g for 45 min (1.083 g ltr^{-1} Histopaque (Sigma–Aldrich, UK)). Cells were washed three times 600 g for 8 min and counted by Tryphan Blue exclusion (0.1% v/v Sigma–Aldrich, UK).

2.3. Isolation of monocytes and generation of monocyte-derived dendritic cells

$CD14^{+}$ cells were isolated using magnetic assisted cell sorting (MACS) as previously described (Yamakawa et al., 2008), PBMCs were incubated with anti human CD14 microbeads (Miltenyi Biotech, Germany) and placed over an optimised LS column (Miltenyi Biotech, Germany). $CD14^{+}$ cells were used to generate moDC and the remaining, flow through, $CD14^{-}$ PBMC were retained for subsequent isolation of lineage negative cells. $CD14^{+}$ cells were resuspended in tissue culture medium (TCM) supplemented with recombinant bovine (rbo) IL-4 and rboGM-CSF (both RVC) to 1×10^{6} cells ml^{-1} and cultured for 7 days at 37 °C and 5% CO_2 (Yamakawa et al., 2008) with 2 ml of media replenished on days 2 and 5. Resultant moDC were harvested and used as described in IFNα/β stimulation assays.

2.4. Generation of Lineage Negative (LIN^{-}) cell isolation by negative selection

As pDC in other species were shown to be lineage negative (McKenna et al., 2005; Siegal et al., 1999), $CD14^{-}$ PBMCs were incubated with a cocktail of antibodies to bovine lineage-specific markers to allow MACS isolation of LIN^{-} cells. Cells were incubated with 10 μl of antibody per 18×10^{7} $CD14^{-}$ PBMCs for 15 min at 4 °C in PBS/1% BSA (CD3 (MM1A, VMRD, USA); used at 10 μg ml^{-1} in PBS/1% BSA, CD2 (CC42), CD4 (CC30), CD8 (CC63), CD21 (CC21) and sIgM (ILA-58; used neat from hybridoma cell culture supernatant)). Cells were washed twice and incubated with 10 μl per 1×10^{7} cells of goat anti-mouse IgG labelled microbeads (Miltenyi Biotech, Germany) for 15 min at 4 °C, washed and placed over 2 successive, optimised LS column (Miltenyi Biotech, Germany) and LIN^{-} cells collected. The resultant LIN^{-} cells were used for subsequent characterisation as described.

2.5. Phenotypic characterisation by Fluorescent Activated Cell Sorting (FACS)

Isolated LIN^{-} cells were stained in 96 well plates (Greiner, Germany) at 2.5×10^{5} cells per well in FACS buffer (PBS/1% BSA/0.1% NaN_3). Cells were blocked with goat serum and incubated with 25 μl of primary antibody (Table 1) diluted in FACS buffer for 15 min at room temperature, washed with FACS buffer and incubated with a 25 μl of fluorescein labelled goat anti-mouse IgG secondary antibody for 15 min at room temperature in the dark. Stained cells were washed and resuspended in FACS buffer and analysed by FACS using a FACSAria (BD Immunocytometry Systems) and analysed with FlowJo 7.1 webstart software (TreeStar Inc, Ashland, USA). Data for 5,000 gated events for each sample were collected post gating (Fig. 2 and Supplementary Data Fig. 1)).

Table 1
Antibody Panel Used For Surface Antigen Expression Determination.

Marker	Antibody	Reference	Isotype	Staining
CD1b[b]	CC14	(Howard et al., 1991)	IgG_1	0/3
CD1w3[b]	CC43	(Howard et al., 1991)	IgG_{2b}	0/3
CD2[a,b]	CC42	(Koyama et al., 1991)	IgG_1	0/3
CD3[a,b]	MM1A	[c]	IgG_1	0/3
CD4[a,b]	CC30	(Howard and Naessens, 1993)	IgG_1	0/3
CD5[b]	CC17	(Howard and Naessens, 1993; Letesson et al., 1991)	IgG_1	0/3
CD6[b]	CC38	(Letesson and Bensaid, 1991)	IgG_{2b}	2/3
CD8[a,b]	CC58	(Howard and Naessens, 1993)	IgG_1	0/3
WC1[b]	CC15	(Crocker et al., 1993)	IgG_{2a}	3/3
HLA-DQ[a,b]	CC158	[d]	IgG_1	2/3
HLA-DR[a,b]	ILA21	(Naessens et al., 1990)	IgG_{2a}	3/3
CD11a[b]	ILA99	(Howard et al., 1991)	IgG_{2a}	2/3
CD11b[b]	CC94	(Howard et al., 1991)	IgG_1	0/3
CD11c[b]	ILA16	(Brackenbury et al., 2005)	IgG_1	0/3
CD45RO[b]	ILA116	(Ballingall et al., 2001)	IgG_3	3/3
CD45RB[b]	CC76	(Howard and Naessens, 1993)	IgG_1	0/3
CD62L[b]	CC32	(Howard and Naessens, 1993; Howard et al., 1992)	IgG_1	0/3
CD172a[b]	CC149	(Brooke et al., 1998)	IgG_{2b}	1/3
CD205[b]	CC98	(Howard and Naessens, 1993)	IgG_{2b}	0/3
CD80[b]	N32/52-3	(Bastos et al., 2007)	IgG_1	0/3
CD86[b]	IL-A190	(Bastos et al., 2007)	IgG_1	1/3
CD40[b]	IL-A158	(Bastos et al., 2007)	IgG_1	0/3
CD21[b]	CC21	(Naessens et al., 1990)	IgG_1	0/3
CD26[b]	CC62	(Howard and Naessens, 1993)	IgG_{2b}	0/3
CD14[a,b]	CCG33	(Sopp et al., 1996)	IgG_1	0/3
CD32[b]	CCG36	(Zhang et al., 1995)	IgG_1	0/3
γδTCR[b]	GB12a	(Rhodes et al., 1999)	IgG_{2b}	0/3
CC84 ag[b]	CC84	[d]	IgG_1	0/3
IgM[a]	ILA-58	(Naessens et al., 1990)	IgG_{2a}	–
CD45pan[a]	CC171	(Howard and Naessens, 1993)	IgG_{2a}	–
NKp46[a]	NKp46	(Storset et al., 2004)	IgG_1	–

[a] Denotes antibody panel 1.
[b] Denotes antibody panel 2.
[c] VMRD Inc, Pullman, USA.
[d] Howard et al, unpublished.

2.6. Morphological analysis of cells by SEM and TEM

For scanning electron microscopy (LIN^- and moDC) cells were collected onto poly-L-Lysine coated glass coverslips and fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4. Samples were post-fixed with 1% osmium tetraoxide (Os04) in 0.1 M sodium cacodylate buffer at pH 7.4, dehydrated in a graded series of ethanol and subsequently air dried with the aid of the transitional solvent, hexamethyldisalazane (HMDS). Glass discs containing the cells were mounted onto aluminium stubs and coated with a gold/palladium conducting source on an EMITECH K550 Sputter Coater. Samples were analysed with a JEOL 5500LV Scanning Electron Microscope. For transmission electron microscopy, cell pellets were prepared with Karnovsky-fixative (2% paraformaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4). Post fixation was conducted with 1% (Os04) in 0.1 M sodium cacodylate buffer at pH 7.4. Samples were then en-bloc stained with 2% uranyl acetate in 0.1 M sodium acetate buffer. The cell pellets were subsequently dehydrated in a graded series of ethanol and infiltrated in a propylene oxide-resin mixture. Araldite resin was used for infiltration and final embedding prior to ultramicrotomy. Ultrathin sections were cut at 70 nm thickness and collected onto copper grids. The sections were counter stained with lead citrate and examined at 80 kV with a Philips CM12 Transmission Electron Microscope.

2.7. Analysis for TLR mRNA expression by isolated cells

Total RNA was extracted from lysed cells using the RNeasy mini kit (Qiagen, Chatsworth, CA) according to the manufacturer's protocol and treated with RNase-free DNase I (Ambion (Europe) Ltd., Cambridge, UK). RNA yields and quality were determined using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA (100 ng) was subsequently transcribed to cDNA using the Superscript II reverse transcription system (Promega, Southampton, UK) according to the manufacturer's protocol. cDNA yields and quality were determined using a spectrophotometer as described above. Standard PCR amplifications for each sample (cDNA adjusted to 100 ng μl^{-1}) were carried out using the primers listed on Table 3 with an annealing temperature of 55 °C using a GStorm Thermocycler (Thistle Scientific, UK). These primers were based on bovine sequences with their correct amplicons confirmed by sequencing.

2.8. Stimulation of IFNα/β stimulation assay

pDC in other species have been described to be the major producers of IFNα/β (Hubert et al., 2004; Siegal et al., 1999; Summerfield et al., 2003). PBMCs, monocytes, moDC and LIN^- cells were cultured in TCM in 96 well plates at 5×10^5 cells per well and stimulated with synthetic bacterial DNA in the form of CpG motifs at a final concentration of 50 μg ml^{-1} (Table 2, TIB MolBiol, Germany). Supernatants were collected at 0, 4, 12, 24, 36 and 48 h after stimulation for CpG 2006, CpG 2007 and their respective control sequences and at 48 h only for CpG 2336 and control CpG 2243. IFNα/β was measured using an Mx promoter-chloramphenicol acetyltransferase (Mx-CAT) reporter assay as previously described with some minor modifications (Charleston et al., 2001). Supernatants from stimulated cells as described above were used to induce reporter activity expression within Mx-CAT transfected cells in 24 well plates. CAT expression was analysed with CAT ELISA (Roche Diagnostics, Germany) according manufacturer's instruction after lysing transfected cells with a freeze/thaw cycle.

2.9. Statistical analysis

Statistical analysis was performed using SPSS Version 17 (SPSS Inc, USA) by means of 2-way ANOVA. Post-hoc analyses for multiple significance test comparisons were provided by Bonferroni correction. Graphical representations of data display mean values for each data set ± SD due to skewing towards the right of normal distributions as can be expected from groups of small sizes (n = 4). However, general assumptions of normal data distribution and random data set scatter were confirmed using residual and predicted values to ensure assumptions of the general linear model were correct. Significance values were set at 0.05 for 95% confidence intervals.

3. Results

3.1. Phenotype of LIN^- cells isolated from peripheral blood

As pDCs described in other species lack expression of lineage markers, LIN^- cells were isolated from bovine peripheral blood.

Table 2
CpG ODN sequences

CpG	Sequence
CpG 2006	T∗C∗G∗T∗C∗G∗T∗T∗T∗T∗G∗T∗C∗G∗T∗T∗T∗T∗G∗T∗C∗G∗T∗T
CpG 2006(K)	T∗G∗C∗T∗G∗C∗T∗T∗T∗T∗G∗T∗G∗C∗T∗T∗T∗T∗G∗T∗G∗C∗T∗T
CpG 2007	T∗C∗G∗T∗C∗G∗T∗T∗G∗T∗C∗G∗T∗T∗T∗T∗G∗T∗C∗G∗T∗T
CpG 2007(K)	T∗G∗C∗T∗G∗C∗T∗T∗G∗T∗G∗C∗T∗T∗T∗T∗G∗T∗G∗C∗T∗T
CpG 2336	G∗G∗GGACGACGTCGTGG∗G∗G∗G∗G∗G
CpG 2243	G∗G∗G∗GGAGCATGCTGG∗G∗G∗G∗G∗G

(K) Denotes scambled control sequence.
∗Denotes phosphorothioate bond.

Table 3
Bovine TLR Primer Sequences

Gene (accession number)	Sense primer	Anti-sense primer
Bovine TLR1 (NM_001046504)[a]	TTCCAGAGCTGCCAGAAGAT	GAGATTGTGGTGGGCAAAGT
Bovine TLR2 (NM_174197)[a]	CAGCAACTGAAGACGTTGGA	CACCACTCGCTCTTCACAAA
Bovine TLR3 (NM_001008664)[a]	CCCCAGTCTCACAGAGAAGC	CCTGTGAGTTCTTGCCCAAT
Bovine TLR4 (NM_174198)[a]	TGCTGGCTGCAAAAAGTATG	TCTGCAGGACGATGAAGATG
Bovine TLR5 (NM_001040501)[a]	TGCATCCAGATGCTTTTCAG	CCTTCAGCTCCTGGAGTGTC
Bovine TLR6 (NM_001001159)[a]	AGGCCAAGTATCCAGTGACG	GAGATTGTGGTGGGCAAAGT
Bovine TLR7 (NM_001033761)[a]	GGAAATTGCCCTCGTTGTTA	TGCAGTGTTTCAAGGACCTG
Bovine TLR8 (NM_001033937)[a]	TTGATGACGATGCTGCTTTC	GGGTTACCCCCTAGTTCCAA
Bovine TLR9 (NM_183081)[a]	CAAGTGCTCGACCTGAGTGA	CCATGGTACAGGTCCAGCTT
Bovine TLR10 (NM_001076918)[a]	CACCTGACATCTTTGCGAGA	TTCCCTCATGAAGGCAAATC

[a] (Werling et al., 2006, Provisional RefSeq Accesssion Numbers from NCBI).

LIN$^-$ cells were isolated by initial positive depletion of CD14$^+$ monocytes and subsequent exclusion of CD2$^+$, CD3$^+$, CD4$^+$ and sIgM$^+$ cells. The remaining cells were stained with antibody panel 1 (Table 1) to ensure depletion of lineage positive cells (T cells, B cells, NK cells and monocytes) and determine purity (Fig. 1). In addition, to account for the possiblity of masked epitopes by isolating antibodies, alternative antibody clones for the detection of CD2, CD3, CD4 and sIgM as well as pan T and pan B cell antibodies were tested to determine purity of LIN$^-$ cells (data not shown). All subsequent LIN$^-$ cell isolations for phenotype analysis and functional characteristics were performed using this protocol, ensuring utilisation of enriched LIN$^-$ cells.

To determine the surface phenotype of bovine LIN$^-$ cells, isolated cells from three animals were stained with antibody panel 2 (Table 1). pDCs are thought to be of a lympoid origin, expressing both lymphoid and myeloid markers. Indeed, as shown in Fig. 2, LIN$^-$ cells stained positive for the surface antigens WC1 (a pan γ/δ T cell marker), BoLA-DR, and CD45RO. In 2 out of 3 animals, cells stained also positive for the surface antigens CD6, BoLA-DQ and CD11a, whereas positive staining for surface antigens CD172a, CD80 and CD86 was only found in 1 out of 3 animals. Furthermore, LIN$^-$ cells isolated from all three animals did not stain with antibodies directed against other surface markers shown in Table 1, relative to isotype control staining (Supplementary Data, Fig. 1).

3.2. Morphology of LIN$^-$ cells

pDCs, belonging to the lymphoid lineage in other species, have been described to show a lymphoid morphology until activation, and thus a large nucleus and limited cytomplasm is therefore characteristic of this cell type (Asselin-Paturel et al., 2001; Facchetti et al., 2003). SEM and TEM analysis were employed to ascertain the morphology of the LIN$^-$ cell population isolated as described. Indeed, an enriched population of cells with lymphoid morphology, membrane ruffles and pseudopodia were observed by SEM (Fig. 3a). TEM revealed extensive rough endoplasmic reticulum and golgi apparatus (Fig. 3b), both typical cytoplasmic constituents of pDC aiding cytokine synthesis and rapid activation (Colonna et al., 2004).

3.3. Only LIN$^-$ cells produce IFNα/β in response to Type A and Type B CpG ODN

In the human and murine system, pDC, in contrast to myeloid DC have been characterised by the expression of TLR9, enabling these cells to respond to CpG ODN (Kadowaki et al., 2001). To analyse the response of immune cell subsets in the bovine system, the Type B CpG (CpG B), 2006 and 2007, were investigated initially, based on published information in the ruminant system (Mena et al., 2003). The Type A CpG (CpG A), 2336, was included for comparative reasons, as it has been described before as a potent inducer of IFNα/β production in human pDC as well as in newborn lambs (Nichani et al., 2006). PBMC, monocytes and LIN$^-$ cells were stimulated with 50 $\mu g\,ml^{-1}$ of CpG 2006 and 2007, and their respective scrambled control sequences, 2006K and 2007K over a 48 h period (Fig. 4). Supernatants were collected as described and analysed for IFNα/β production. LIN$^-$ cells responded to CpG 2006 in a time dependent manner, with a increase above controls starting from 12 h, showing a significant difference in IFN production by 48 h compared to PBMC and monocytes. A similar response was observed for CpG 2007 from 24 h, albeit of a lower magnitude. Control sequences, CpG 2006K and 2007K, induce low IFNα/β production as expected, however even by 48 h both induce significant levels in LIN$^-$ cells compared to PBMC and monocytes. IFNα/β induced by CpG 2006 in LIN$^-$ cells was found to be significantly greater than CpG 2006K at 48 h but not for CpG 2007. In contrast, neither total PBMCs nor monocytes produced IFNα/β in response to either CpG ODN over the 48 h period. Having established that 48 h post stimulation showing the highest value for IFNα/β production, we next compared the ability of monocytes, moDC and LIN$^-$ cells in their ability to produce IFNα/β in response to CpG 2336 and its scrambled control CpG 2243. Similar as seen before, LIN$^-$ cells, but not monocytes or moDC produced significantly amounts of IFNα/β (Fig. 5). Control CpG did not induce notable amounts of IFNα/β in any cell type.

3.4. TLR expression of LIN$^-$ cells

The characteristic ability of pDCs to produce large amounts of IFNα/β in response to viral and bacterial stimuli (CpG ODN) is owed to their expression of TLR7 and 9. Using primers specific for bovine TLRs 1–10, the repertoire of TLRs expressed by unstimulated LIN$^-$ cells from 4 animals at the mRNA level was investigated by PCR. LIN$^-$ cells express an array of TLRs at the mRNA level of which there is animal to animal variation observed. LIN$^-$ cells express TLR3, TLR4 and TLR6 in all animals, TLR1 in 3 animals and TLR2, TLR7 and TLR10 in 2 animals (Fig. 6, Table 3). Positive plasmid controls for bovine TLRs 1–10 were included in each reaction.

4. Discussion

Thus far, bovine DCs are largely subdivided into bone marrow derived DCs (BMDC), moDCs and ALVC based upon their anatomical location and surface antigen expression (Hope et al., 2006; Hope et al., 2000; Yamakawa et al., 2008). Similar to human moDCs, bovine moDCs express myeloid markers CD11a, CD11b, CD14 and CD172a, up-regulate CD40, CD80 and CD86 upon activation

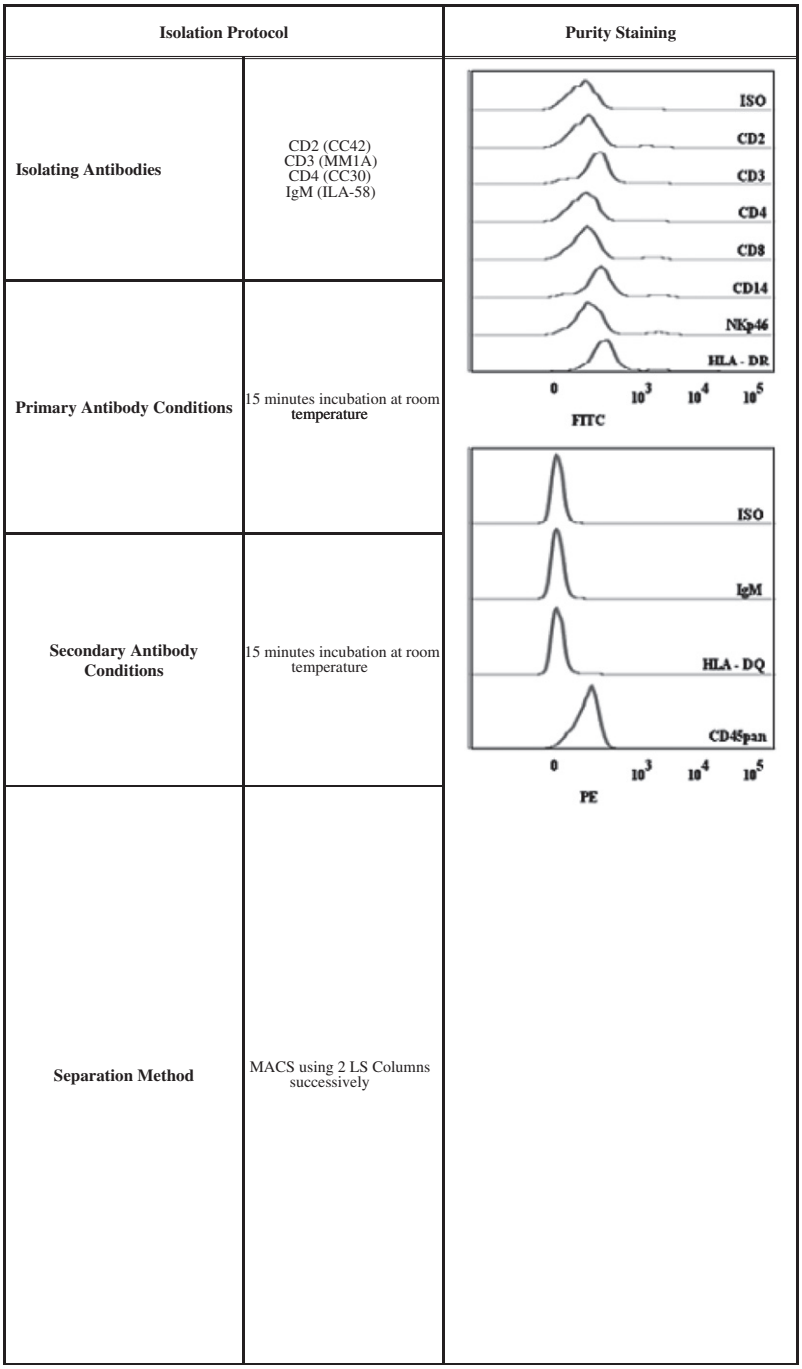

Fig. 1. Sorting protocol for bovine lineage negative cells Isolated LIN$^-$ cells were stained with antibody Panel 1 (Table 1: antibody subset (a)). Data shown is representative of a single population with 5000 events recorded within the stopping gate and is displayed by overlaying histograms of cell count against fluorescence intensity of FITC and PE against control, respectively with 100% offsetting.

and are efficient antigen presenting cells (Werling et al., 1999). AVLC are essentially defined of myeloid origin and are easily distinguishable by flow cytometry from other afferent lymph cells by the expression of CD205 (Howard and Hope, 2000). As displayed in Fig. 1 and Fig. 2, cells isolated in the present study exhibit an enriched population of PBMC uniform in lacking lineage positive markers, however are heterogenous for those markers indicating phenotype (WC1, CD6, CD11a, CD26, CD172a and CD45RO) indicating that LIN$^-$ cells contain a subpopulation of which represents bovine pDC. Additionally, varying expression levels between animals could be a result of differing underlying activation status of circulating cells upon isolation. LIN$^-$ isolated in the present study are distinct from moDC and ALVC populations, primarily by the expression of WC1 and the lack of CD40, CD80 and CD86 expression (Fig. 2). Prior to maturation, pDCs are understood to be poor stimulators of naïve T cells, however, they have been reported to up-regulate expression of co-stimulatory molecules on activation (Mouries et al., 2008). Expression of MHC class II (HLA-DQ and HLA-DR) and CD45RO by the cells isolated in the present study indicated a cell type of lymphoid origin, whereas CD11a and CD172a point to a myeloid cell type. This expression pattern is, however, in line with pDC described in other species (Cella et al.,

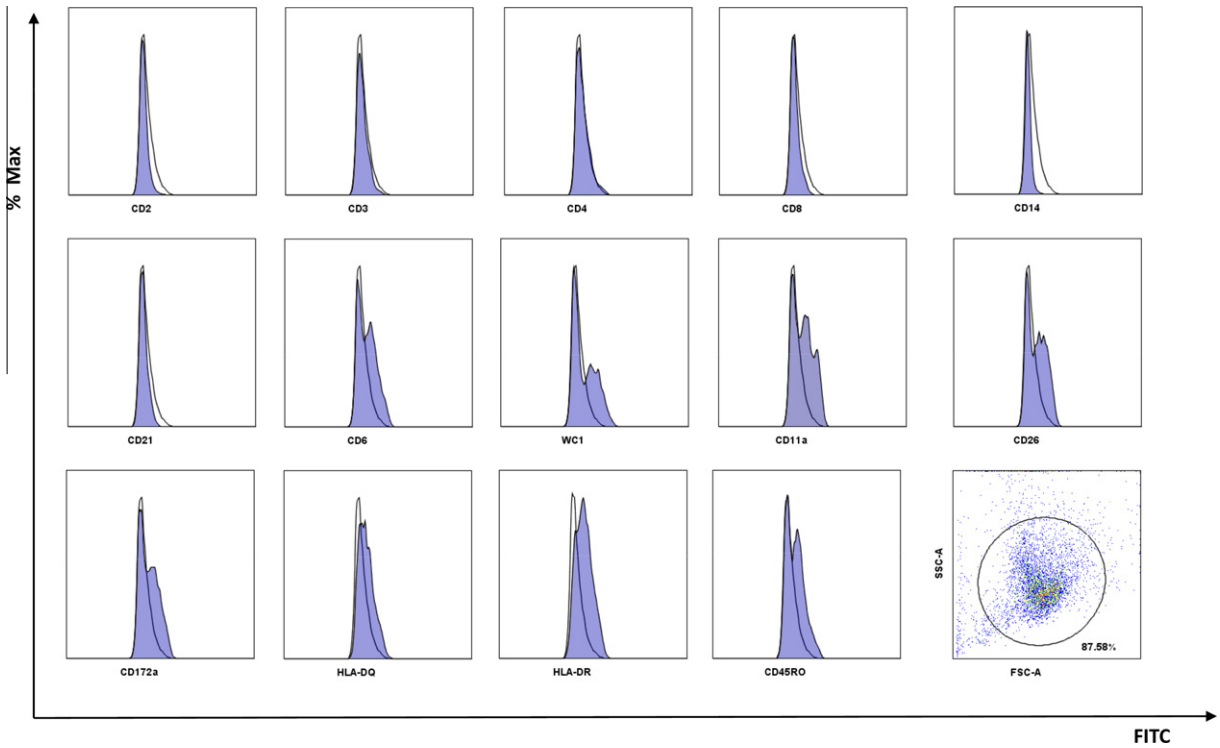

Fig. 2. Surface phenotype of bovine lineage negative cells LIN$^-$ cells were examined for surface antigen expression with antibodies described in Panel 2 (Table 1: antibody subset (b)). Each surface marker (Blue) is shown relative to isotype control staining (Empty). Analysis was performed on 5000 gated events as shown in lower right lot. Graphical displays are sample (blue filled) and control (empty) overlaid histograms with cell count versus fluorescent intensity with 0% offsetting. Data is representative of 3 individual experiments from different animals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1999; Colonna et al., 2004; Fitzgerald-Bocarsly, 2002; Hubert et al., 2004; Masten et al., 2006; Pascale et al., 2008; Summerfield et al., 2003). Therefore, the phenotype of LIN$^-$ cells described herin, as well as the recent finding that CD45 is required for the production of IFNα/β, suggests that the enriched cell population described is likely to contain the bovine counterparts to human pDCs (Montoya et al., 2006).

An important anti viral response is the rapid production of IFNα/β inducing an active anti viral state in both, autocrine and paracrine manner. It is clear that pDCs are not the only IFNα/β producing cells (Diebold et al., 2003), however, their unique ability to secrete large amounts of IFNα/β depends upon cellular sensors and subsequent coupling of recognition to IFNα/β synthesis. pDCs have been shown to express TLR7, TLR8 and TLR9, and their ligation induces IFNα/β production (Hornung et al., 2002). TLR7 and TLR8 respond mainly to ssRNA, although there are reports of TLR7 responding to CpG stimulation (Hornung et al., 2002). TLR9 recognises unmethylated DNA containing CpG motifs, which are present in bacterial and DNA viral genomes. We show in this study that bovine enriched LIN$^-$ cells produce significantly larger (100–1000 fold) amounts of IFNα/β in response to stimulatory CpG ODN sequences compared to PBMC, monocytes and moDC (Fig. 4 and Fig. 5), similar to that described for other veterinary species (Guzylack-Piriou et al., 2004; Pascale et al., 2008; Summerfield et al., 2003). LIN$^-$ cells respond similarly to Type A and Type B CpG ODN (CpG A and CpG B), an observation not shared by other ruminants (Guzylack-Piriou et al., 2004; Mena et al., 2003; Pascale et al., 2008). CpG A, such as CpG 2216 in the present study, contain stimulatory sequences linked through phosphorothioate bonds flanked by protective poly G strands and are targeted to endocytic compartments enabling potent TLR9-IRF7 dependent IFNα/β induction (Honda et al., 2005). Synthetic CpG motifs mimic the stimulatory effects of both bacterial DNA and viral replication intermediates signalling through TLR9 resulting in the activation and nuclear translocation of transcription factors, NFκB and AP-1 (Takeshita et al., 2004). Conventional CpG B lack poly G strands and potent GC dinucleotide rich sequences and are promptly transported to lysosomal compartments, inducing pro-inflammatory cytokines through a TLR9-IRF5 dependent pathway (Kerkmann et al., 2003). CpG B used herein (CpG 2006 and CpG 2007) resulted in differing IFNα/β stimulation levels in LIN$^-$ cells (Fig. 4). CpG 2006 contains 3 separate 'GTCGTT' stimulatory sequences whereas CpG 2007 does not, (Table 2) possibly explaining increased IFNα/β stimulation by CpG 2006 compared to CpG 2007. Such a stimulatory motif was shown to be required for activation of bovine leukocytes and is known to be a potent inducer of IFNα/β in human pDC (Pontarollo et al., 2002; Zhang et al., 2001). Scrambled control sequences used in the current study also induce low but significant (CpG B controls only) levels of IFNα/β from LIN$^-$ cells at 48 h compared to PBMC and monocytes, although not a characteristically 100–1000 fold increase compared to other cell types, this indicates that even in a scrambled format the control sequences exhibit low level stimulatory capacity.

We attempted to assess the percentage of cells producing IFNα/β using bovine specific IFNα antibodies previously reported for use within bovine and ovine tissue sections (Brackenbury et al., 2005; Nichani et al., 2006). Our attempts via flow cytometry and fluorescent microscopy provided limited insights (data not shown); however others have described these clones as unsuitable for flow cytometry [(Brackenbury et al., 2005) and I.Cornil-Schwartz, personal communication]. Therefore due to the lack of reagents, we were unable in the present study to assess the percentage of cells within the isolated population producing IFNα/β; however Griebel *et al* observed a substantial increase in production of IFNα/β when PBMCs were depleted of CD3$^+$, CD14$^+$ and CD21$^+$ cells and subsequently cultured with the Class A CpG ODN 2216 (Griebel et al., 2005). LIN$^-$ in the present study did not express TLR8 or 9, as assessed by RT-PCR, although in the case of TLR8 in 'resting state'

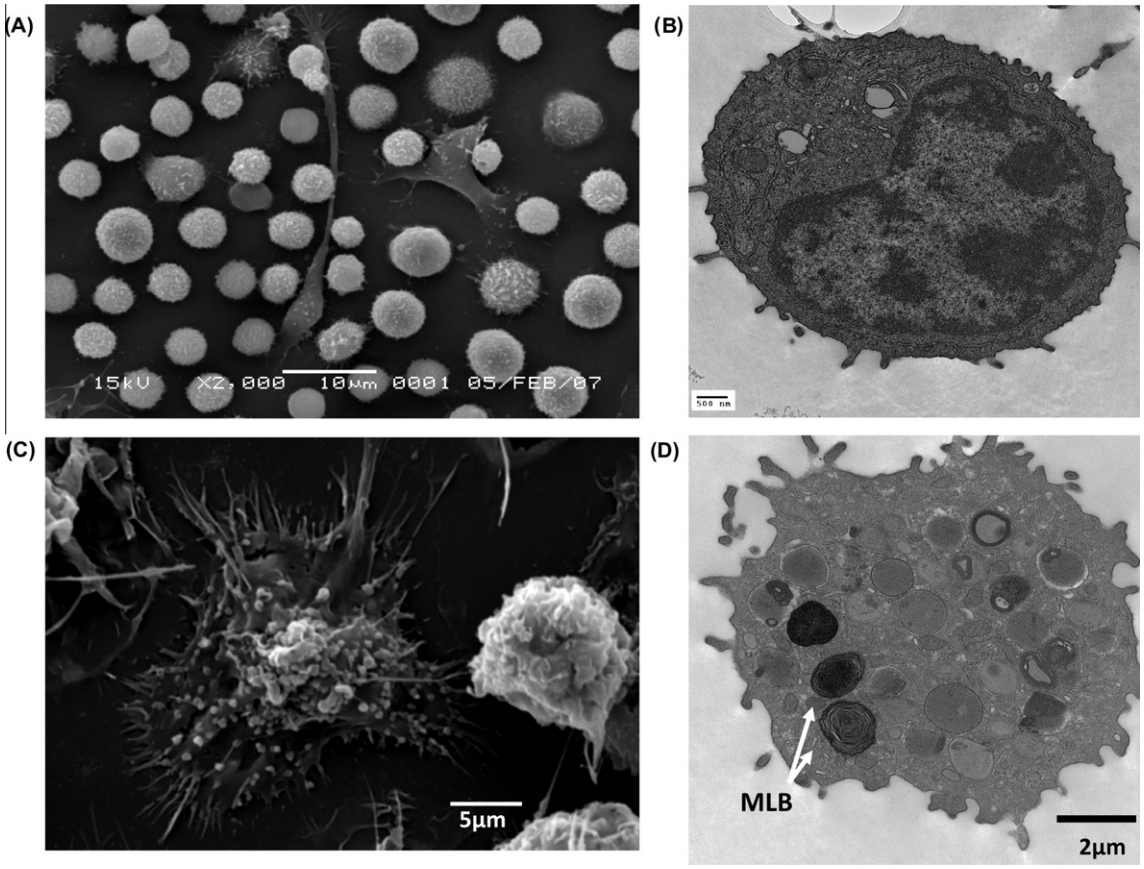

Fig. 3. Morphology of bovine lineage negative cells LIN$^-$ cells were prepared for both SEM and TEM from freshly isolated cells. An enriched population can be observed by SEM (A) with lymphoid morphology and membrane ruffles. TEM (B) displays cytoplasmic characteristics of pDCs with extensive ER and Golgi apparatus. For comparison bovine moDC were also prepared for SEM (C) and TEM (D) as described. MLB = Multilammellar bodies (Magnification: x 15,000).

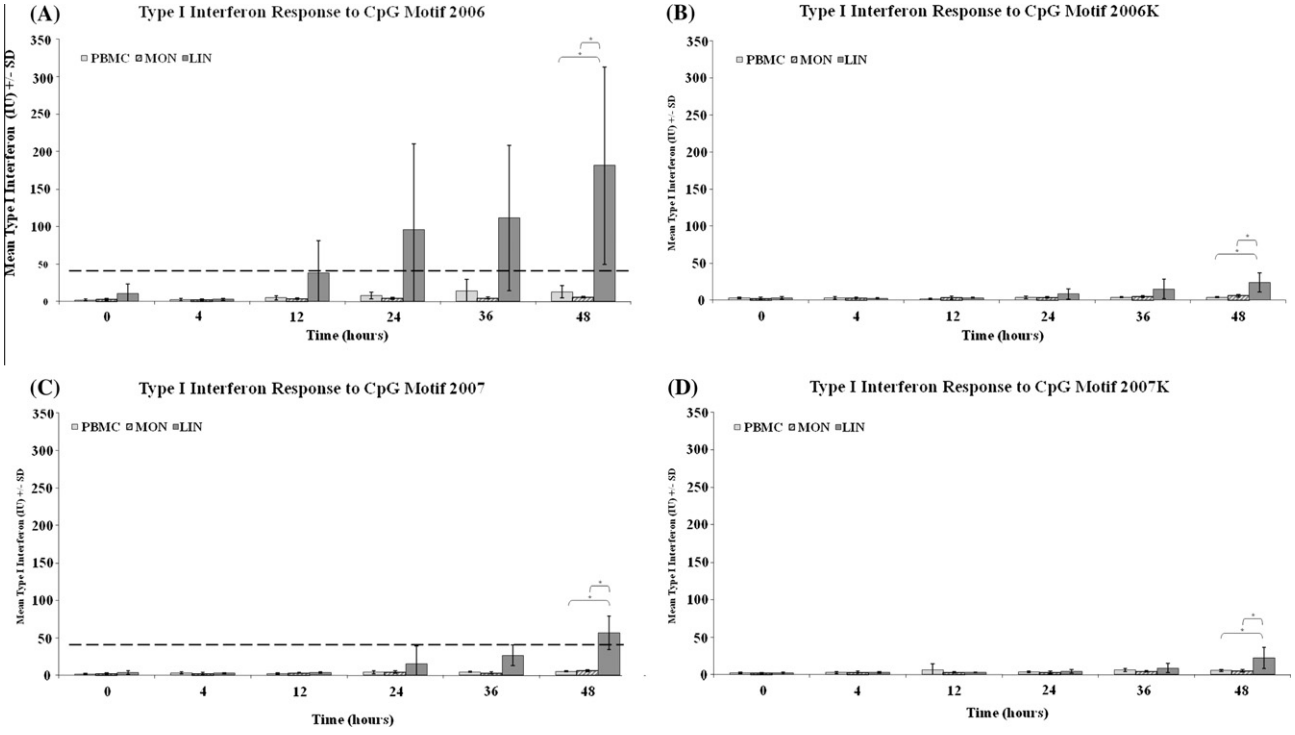

Fig. 4. Type I interferon response to CpG ODN PBMC, monocytes (MON) and LIN$^-$ cells (LIN) were isolated from the same animal and stimulated with 50 µg ml^{-1} of CpG 2006 (A) CpG 2006K (B) CpG 2007 (C) and CpG 2007K (D) for 48 h. Supernatants were collected at various time points and assayed for presence of IFNα/β (n = 4). Maximal response to controls CpG 2006K and CpG 2007K is displayed by grey dashed line in graph (A) and (C) respectively. *p-value < 0.05 at 95% confidence interval.

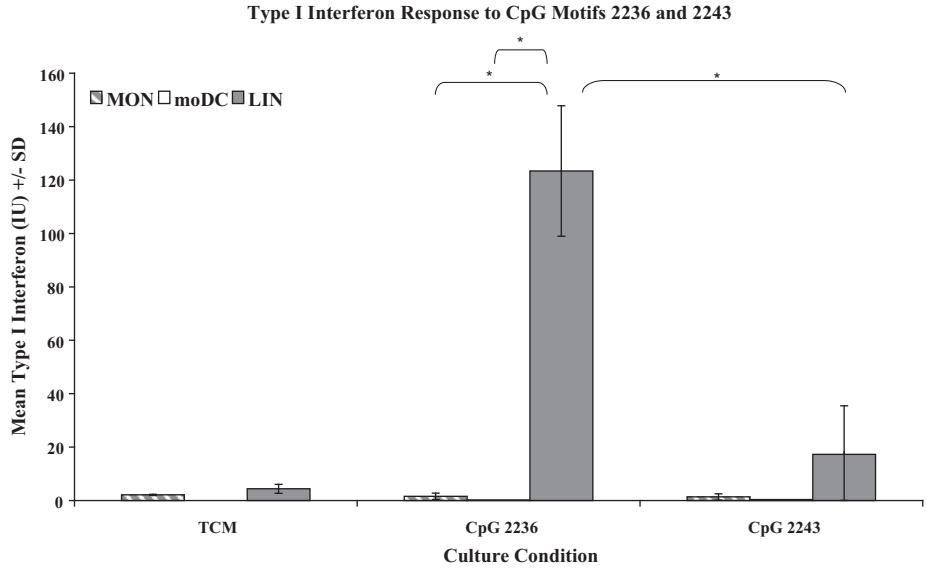

Fig. 5. Type I interferon response to CpG 2336 and 2243 Monocytes (MON), moDC and LIN$^-$ cells (LIN) were isolated from the same animal and stimulated for 48 h with media alone, 50 μg ml^{-1} CpG 2336 and 50 μg ml^{-1} control CpG 2243. Supernatants were harvested and analysed for the presence of IFNα/β (n = 4). $^*p$-value < 0.05 at 95% confidence interval.

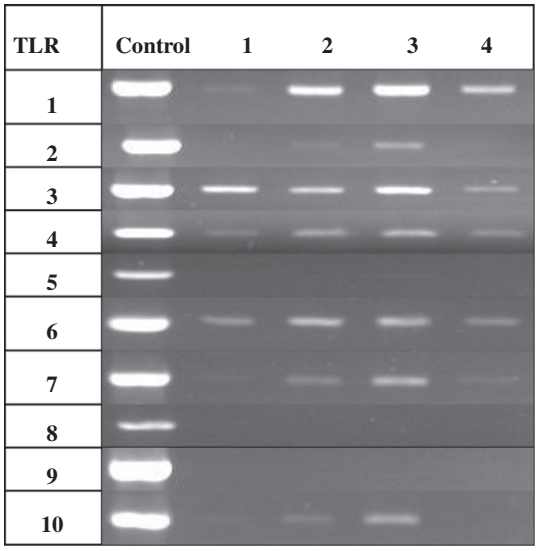

Fig. 6. Expression of TLR in Lineage Negative Cells at mRNA Level cDNA of LIN$^-$ cells isolated from 4 animals (Lanes 1 through 4) were screened for the presence of bovine TLR1-10 by PCR using primers described in Table 3. Products are analysed after 35 cycles by gel electrophoresis on 1% agarose gels stained with 0.005% SafeView nucleic acid dye (NBS Biologicals, UK). Each TLR set contained a plasmid positive control and ran alongside a 1 kb DNA ladder (O'GeneRuler, Fermentas, Germany).

pDC this is not surprising (Hornung et al., 2002; Hubert et al., 2004). Transcripts for TLR7 mRNA were only observed in 2 animals further indicating that isolated LIN$^-$ cells contain a subset of cells pertaining to bovine pDC as suggested by FACS phenotype data (Fig. 1 and Fig. 2). Expression of TLR7 suggests that synthetic ODN could also act via TLR7 in cattle (Fig. 6) as reported in humans elsewhere (Hornung et al., 2002). Lack of TLR9 expression might be due to post-translational activation, and as such may not be constitutively expressed at the mRNA level (Jurk et al., 2006; Park et al., 2008). Nevertheless, the TLR expression pattern in bovine LIN$^-$ cells is distinct from that described for other bovine APC (Werling et al., 2006). In addition to these differences in response to CpG ODN, we were also able to demonstrate specific IFNα/β responses from LIN$^-$ cells compared to various bovine immune cell subsets in their response to different non cytopathic BVDV strains (Gibson et al., 2011).

In the present study, we provide initial information towards the identification of so far uncharacterised circulating bovine pDC. Phenotype analysis of LIN$^-$ cells described these cells as distinct from other bovine DC subsets containing a population expressing both lymphoid and myeloid surface markers. Functionally, LIN$^-$ cells produce significant amounts of IFNα/β in response to CpG ODN compared to PBMC, monocytes and moDC. Taken together, data presented herein suggests that isolation of bovine LIN$^-$ cells has resulted in an enriched cell population. This enriched population are uniquely capable of producing IFNα/β in response to CpG ODN and therefore likely contain bovine pDC.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2011.05.002.

References

Asselin-Paturel, C., Boonstra, A., Dalod, M., Durand, I., Yessaad, N., Dezutter-Dambuyant, C., Vicari, A., O'Garra, A., Biron, C., Briere, F., Trinchieri, G., 2001. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. Nat. Immunol. 2, 1144–1150.

Baigent, S.J., Goodbourn, S., McCauley, J.W., 2004. Differential activation of interferon regulatory factors-3 and -7 by non-cytopathogenic and cytopathogenic bovine viral diarrhoea virus. Vet. Immunol. Immunopathol. 100, 135–144.

Baigent, S.J., Zhang, G., Fray, M.D., Flick-Smith, H., Goodbourn, S., McCauley, J.W., 2002. Inhibition of beta interferon transcription by noncytopathogenic bovine viral diarrhea virus is through an interferon regulatory factor 3-dependent mechanism. J. Virol. 76, 8979–8988.

Ballingall, K.T., Waibochi, L., Holmes, E.C., Woelk, C.H., MacHugh, N.D., Lutje, V., McKeever, D.J., 2001. The CD45 locus in cattle: allelic polymorphism and evidence for exceptional positive natural selection. Immunogenetics 52, 276–283.

Bastos, R.G., Johnson, W.C., Brown, W.C., Goff, W.L., 2007. Differential response of splenic monocytes and DC from cattle to microbial stimulation with Mycobacterium bovis BCG and Babesia bovis merozoites. Vet. Immunol. Immunopathol.115, 334–345.

Brackenbury, L.S., Carr, B.V., Stamataki, Z., Prentice, H., Lefevre, E.A., Howard, C.J., Charleston, B., 2005. Identification of a cell population that produces alpha/beta interferon in vitro and in vivo in response to noncytopathic bovine viral diarrhea virus. J. Virol. 79, 7738–7744.

Brooke, G.P., Parsons, K.R., Howard, C.J., 1998. Cloning of two members of the SIRP alpha family of protein tyrosine phosphatase binding proteins in cattle that are expressed on monocytes and a subpopulation of dendritic cells and which mediate binding to CD4 T cells. Eur. J. Immunol. 28, 1–11.

Cella, M., Jarrossay, D., Facchetti, F., Alebardi, O., Nakajima, H., Lanzavecchia, A., Colonna, M., 1999. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. Nat. Med. 5, 919–923.

Charleston, B., Fray, M.D., Baigent, S., Carr, B.V., Morrison, W.I., 2001. Establishment of persistent infection with non-cytopathic bovine viral diarrhoea virus in cattle is associated with a failure to induce type I interferon. J. Gen. Virol. 82, 1893–1897.

Colonna, M., Trinchieri, G., Liu, Y.J., 2004. Plasmacytoid dendritic cells in immunity. Nat. Immunol. 5, 1219–1226.

Crocker, G., Sopp, P., Parsons, K., Davis, W.C., Howard, C.J., 1993. Analysis of the gamma/delta T cell restricted antigen WC1. Vet. Immunol. Immunopathol. 39, 137–144.

Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S., Reis e Sousa, C., 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 303, 1529–1531.

Diebold, S.S., Montoya, M., Unger, H., Alexopoulou, L., Roy, P., Haswell, L.E., Al-Shamkhani, A., Flavell, R., Borrow, P., Reis e Sousa, C., 2003. Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. Nature 424, 324–328.

Facchetti, F., Vermi, W., Mason, D., Colonna, M., 2003. The plasmacytoid monocyte/interferon producing cells. Virchows Arch. 443, 703–717.

Fitzgerald-Bocarsly, P., 2002. Natural interferon-alpha producing cells: the plasmacytoid dendritic cells. Biotech. Suppl. 16–20 (22), 19–24.

Gibson, A., Larsson, J., Bateman, M., Brownlie, J., Werling, D., 2011. BVDV Strain and Cell Type Specific Inhibition of Type I Interferon Pathways. J. Virol. 85, 3695–3697.

Griebel, P.J., Brownlie, R., Manuja, A., Nichani, A., Mookherjee, N., Popowych, Y., Mutwiri, G., Hecker, R., Babiuk, L.A., 2005. Bovine toll-like receptor 9: a comparative analysis of molecular structure, function and expression. Vet. Immunol. Immunopathol. 108, 11–16.

Guzylack-Piriou, L., Balmelli, C., McCullough, K.C., Summerfield, A., 2004. Type-A CpG oligonucleotides activate exclusively porcine natural interferon-producing cells to secrete interferon-alpha, tumour necrosis factor-alpha and interleukin-12. Immunology 112, 28–37.

Haller, O., Kochs, G., 2002. Interferon-induced mx proteins: dynamin-like GTPases with antiviral activity. Traffic 3, 710–717.

Honda, K., Ohba, Y., Yanai, H., Negishi, H., Mizutani, T., Takaoka, A., Taya, C., Taniguchi, T., 2005. Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. Nature 434, 1035–1040.

Honda, K., Sakaguchi, S., Nakajima, C., Watanabe, A., Yanai, H., Matsumoto, M., Ohteki, T., Kaisho, T., Takaoka, A., Akira, S., Seya, T., Taniguchi, T., 2003. Selective contribution of IFN-alpha/beta signaling to the maturation of dendritic cells induced by double-stranded RNA or viral infection. Proc. Nat. Acad. Sci. USA 100, 10872–10877.

Hope, J.C., Howard, C.J., Prentice, H., Charleston, B., 2006. Isolation and purification of afferent lymph dendritic cells that drain the skin of cattle. Nat. Protoc. 1, 982–987.

Hope, J.C., Werling, D., Collins, R.A., Mertens, B., Howard, C.J., 2000. Flt-3 ligand, in combination with bovine granulocyte-macrophage colony-stimulating factor and interleukin-4, promotes the growth of bovine bone marrow derived dendritic cells. Scand. J. Immunol. 51, 60–66.

Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdorfer, B., Giese, T., Endres, S., Hartmann, G., 2002. Quantitative expression of toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J. Immunol. 168, 4531–4537.

Hovanessian, A.G., 2007. On the discovery of interferon-inducible, double-stranded RNA activated enzymes: the 2'-5'oligoadenylate synthetases and the protein kinase PKR. Cytokine Growth Factor Rev. 18, 351–361.

Howard, C.J., Hope, J.C., 2000. Dendritic cells, implications on function from studies of the afferent lymph veiled cell. Vet. Immunol. Immunopathol. 77, 1–13.

Howard, C.J., Morrison, W.I., Bensaid, A., Davis, W., Eskra, L., Gerdes, J., Hadam, M., Hurley, D., Leibold, W., Letesson, J.J., et al., 1991. Summary of workshop findings for leukocyte antigens of cattle. Vet. Immunol. Immunopathol. 27, 21–27.

Howard, C.J., Sopp, P., Parsons, K.R., 1992. L-selectin expression differentiates T cells isolated from different lymphoid tissues in cattle but does not correlate with memory. Immunology 77, 228–234.

Howard, C.J., Naessens, J., 1993. Summary of workshop findings for cattle (tables 1 and 2). Vet. Immunol. Immunopathol. 39, 25–47.

Hubert, F.X., Voisine, C., Louvet, C., Heslan, M., Josien, R., 2004. Rat plasmacytoid dendritic cells are an abundant subset of MHC class II+ CD4+CD11b-OX62- and type I IFN-producing cells that exhibit selective expression of Toll-like receptors 7 and 9 and strong responsiveness to CpG. J. Immunol. 172, 7485–7494.

Jamin, A., Gorin, S., Le Potier, M.F., Kuntz-Simon, G., 2006. Characterization of conventional and plasmacytoid dendritic cells in swine secondary lymphoid organs and blood. Vet. Immunol. Immunopathol. 114, 224–237.

Jurk, M., Kritzler, A., Debelak, H., Vollmer, J., Krieg, A.M., Uhlmann, E., 2006. Structure-activity relationship studies on the immune stimulatory effects of base-modified CpG toll-like receptor 9 agonists. Chem. Med. Chem. 1, 1007–1014.

Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R.W., Kastelein, R.A., Bazan, F., Liu, Y.J., 2001. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J. Exp. Med. 194, 863–869.

Keir, M.E., Stoddart, C.A., Linquist-Stepps, V., Moreno, M.E., McCune, J.M., 2002. IFN-alpha secretion by type 2 predendritic cells up-regulates MHC class I in the HIV-1-infected thymus. J. Immunol. 168, 325–331.

Kerkmann, M., Rothenfusser, S., Hornung, V., Towarowski, A., Wagner, M., Sarris, A., Giese, T., Endres, S., Hartmann, G., 2003. Activation with CpG-A and CpG-B oligonucleotides reveals two distinct regulatory pathways of type I IFN synthesis in human plasmacytoid dendritic cells. J. Immunol. 170, 4465–4474.

Koyama, H., Tsunemi, E., Hohdatsu, T., 1991. Preparation and characterization of a monoclonal antibody against bovine T cell surface antigen associated with the bovine homologue of CD2. J. Vet. Med. Sci. 53, 153–157.

Letesson, J.J., Bensaid, A., 1991. Individual antigens of cattle. Bovine CD6 (BoCD6). Vet. Immunol. Immunopathol. 27, 61–64.

Letesson, J.J., Lambot, M., Mulumba, M., Depelchin, A., 1991. Reactivity of the workshop CD5 panel antibodies with the purified CD5 antigen. Vet. Immunol. Immunopathol. 27, 241.

Martinez, J., Huang, X., Yang, Y., 2008. Direct action of type I IFN on NK cells is required for their activation in response to vaccinia viral infection in vivo. J. Immunol. 180, 1592–1597.

Masten, B.J., Olson, G.K., Tarleton, C.A., Rund, C., Schuyler, M., Mehran, R., Archibeque, T., Lipscomb, M.F., 2006. Characterization of myeloid and plasmacytoid dendritic cells in human lung. J. Immunol. 177, 7784–7793.

McKenna, K., Beignon, A.S., Bhardwaj, N., 2005. Plasmacytoid dendritic cells: linking innate and adaptive immunity. J. Virol. 79, 17–27.

Mena, A., Nichani, A.K., Popowych, Y., Ioannou, X.P., Godson, D.L., Mutwiri, G.K., Hecker, R., Babiuk, L.A., Griebel, P., 2003. Bovine and ovine blood mononuclear leukocytes differ markedly in innate immune responses induced by Class A and Class B CpG-oligodeoxynucleotide. Oligonucleotides 13, 245–259.

Meyers, G., Thiel, H.J., 1996. Molecular characterization of pestiviruses. Adv. Virus Res. 47, 53–118.

Montoya, M., Dawes, R., Reid, D., Lee, L.N., Piercy, J., Borrow, P., Tchilian, E.Z., Beverley, P.C., 2006. CD45 is required for type I IFN production by dendritic cells. Eur. J. Immunol. 36, 2150–2158.

Montoya, M., Schiavoni, G., Mattei, F., Gresser, I., Belardelli, F., Borrow, P., Tough, D.F., 2002. Type I interferons produced by dendritic cells promote their phenotypic and functional activation. Blood 99, 3263–3271.

Mouries, J., Moron, G., Schlecht, G., Escriou, N., Dadaglio, G., Leclerc, C., 2008. Plasmacytoid dendritic cells efficiently cross-prime naive T cells in vivo after TLR activation. Blood 112, 3713–3722.

Naessens, J., Newson, J., McHugh, N., Howard, C.J., Parsons, K., Jones, B., 1990. Characterization of a bovine leucocyte differentiation antigen of 145,000 MW restricted to B lymphocytes. Immunology 69, 525–530.

Nichani, A.K., Mena, A., Kaushik, R.S., Mutwiri, G.K., Townsend, H.G., Hecker, R., Krieg, A.M., Babiuk, L.A., Griebel, P.J., 2006. Stimulation of innate immune responses by CpG oligodeoxynucleotide in newborn lambs can reduce bovine herpesvirus-1 shedding. Oligonucleotides 16, 58–67.

Park, B., Brinkmann, M.M., Spooner, E., Lee, C.C., Kim, Y.M., Ploegh, H.L., 2008. Proteolytic cleavage in an endolysosomal compartment is required for activation of Toll-like receptor 9. Nat. Immunol. 9, 1407–1414.

Pascale, F., Contreras, V., Bonneau, M., Courbet, A., Chilmonczyk, S., Bevilacqua, C., Epardaud, M., Niborski, V., Riffault, S., Balazuc, A.M., Foulon, E., Guzylack-Piriou, L., Riteau, B., Hope, J., Bertho, N., Charley, B., Schwartz-Cornil, I., 2008. Plasmacytoid dendritic cells migrate in afferent skin lymph. J. Immunol. 180, 5963–5972.

Pontarollo, R.A., Rankin, R., Babiuk, L.A., Godson, D.L., Griebel, P.J., Hecker, R., Krieg, A.M., den Hurk, S., 2002. Monocytes are required for optimum in vitro stimulation of bovine peripheral blood mononuclear cells by non-methylated CpG motifs. Vet. Immunol. Immunopathol. 84, 43–59.

Rhodes, S.G., Cocksedge, J.M., Collins, R.A., Morrison, W.I., 1999. Differential cytokine responses of CD4+ and CD8+ T cells in response to bovine viral diarrhoea virus in cattle. J. Gen. Virol. 80 (Pt. 7), 1673–1679.

Seeds, R.E., Gordon, S., Miller, J.L., 2006. Receptors and ligands involved in viral induction of type I interferon production by plasmacytoid dendritic cells. Immunobiology 211, 525–535.

Siegal, F.P., Kadowaki, N., Shodell, M., Fitzgerald-Bocarsly, P.A., Shah, K., Ho, S., Antonenko, S., Liu, Y.J., 1999. The nature of the principal type 1 interferon-producing cells in human blood. Science 284, 1835–1837.

Sopp, P., Kwong, L.S., Howard, C.J., 1996. Identification of bovine CD14. Vet. Immunol. Immunopathol. 52, 323–328.

Storset, A.K., Kulberg, S., Berg, I., Boysen, P., Hope, J.C., Dissen, E., 2004. NKp46 defines a subset of bovine leukocytes with natural killer cell characteristics. Eur. J. Immunol. 34, 669–676.

Summerfield, A., Guzylack-Piriou, L., Schaub, A., Carrasco, C.P., Tache, V., Charley, B., McCullough, K.C., 2003. Porcine peripheral blood dendritic cells and natural interferon-producing cells. Immunology 110, 440–449.

Takeshita, F., Gursel, I., Ishii, K.J., Suzuki, K., Gursel, M., Klinman, D.M., 2004. Signal transduction pathways mediated by the interaction of CpG DNA with Toll-like receptor 9. Semin. Immunol. 16, 17–22.

Werling, D., Hope, J.C., Chaplin, P., Collins, R.A., Taylor, G., Howard, C.J., 1999. Involvement of caveolae in the uptake of respiratory syncytial virus antigen by dendritic cells. J. Leukoc. Biol. 66, 50–58.

Werling, D., Hope, J.C., Howard, C.J., Jungi, T.W., 2004. Differential production of cytokines, reactive oxygen and nitrogen by bovine macrophages and dendritic cells stimulated with Toll-like receptor agonists. Immunology 111, 41–52.

Werling, D., Piercy, J., Coffey, T.J., 2006. Expression of TOLL-like receptors (TLR) by bovine antigen-presenting cells-potential role in pathogen discrimination? Vet. Immunol. Immunopathol. 112, 2–11.

Yamakawa, Y., Pennelegion, C., Willcocks, S., Stalker, A., Machugh, N., Burt, D., Coffey, T.J., Werling, D., 2008. Identification and functional characterization of a bovine orthologue to DC-SIGN. J. Leukoc. Biol. 83, 1396–1403.

Zhang, G., Young, J.R., Tregaskes, C.A., Sopp, P., Howard, C.J., 1995. Identification of a novel class of mammalian Fc gamma receptor. J. Immunol. 155, 1534–1541.

Zhang, F., Romano, P.R., Nagamura-Inoue, T., Tian, B., Dever, T.E., Mathews, M.B., Ozato, K., Hinnebusch, A.G., 2001. Binding of double-stranded RNA to protein kinase PKR is required for dimerization and promotes critical autophosphorylation events in the activation loop. J. Biol. Chem. 276, 24946–24958.