Identification and Characterization of Equine Blood Plasmacytoid Dendritic Cells

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Abstract

Dendritic cells (DC) are antigen-presenting cells that can be classified into three major cell subsets: conventional DC1 (cDC1), cDC2 and plasmacytoid DCs (pDC), none of which have been identified in horses. Therefore, the objective of this study was to identify and characterize DC subsets in equine peripheral blood, emphasizing on pDC. Surface marker analysis allowed distinction of putative DC subsets, according to their differential expression of CADM-1 and MHC class II. Equine pDC were found to be Flt3^{+} CD4^{low} CD13^{-} CD14^{-} CD172a^{-} CADM-1^{-}MHCII^{low}. The weak expression of CD4 on equine pDC contrasts with findings in several other mammals. Furthermore, pDC purified by fluorescence-activated cell sorting were found to be the only cell subset able to produce large amounts of IFN-α upon TLR9-agonist stimulation. The pDC identity was confirmed by demonstrating high-levels of *PLAC8*, *RUNX2* and *TCF4* expression, showing pDC-restricted expression in other mammals.

Keywords: horse, dendritic cell subset, plasmacytoid dendritic cell.

Abbreviations: ODN, oligodeoxynucleotides; DC, dendritic cells; cDC, conventional DC; FMO, fluorescence minus one; Flt3, fms-like tyrosine kinase 3; Flt3L, Flt3 ligand; IFN, interferon; MoDC, monocyte-derived DC; pDC, plasmacytoid DC; PBMC, peripheral blood mononuclear cells.

1. Introduction

Dendritic cells (DC) represent the professional antigen-presenting cells of the immune system and are essential regulators of immunity and tolerance (Banchereau and Steinman, 1998). They can be further categorized into conventional DC (cDC), which are responsible for antigen presentation and induction of T-cell responses, and plasmacytoid DC (pDC) representing the most potent type I interferon (IFN) producing cell, able to efficiently sense microbial nucleic acid (Liu, 2005). Conventional DC consist of at least two phenotypically distinct subsets stimulating particular T-cell responses (Schlitzer and Ginhoux, 2014). The murine CD8α^+/ human CD141$^+$ cells comprise the cDC1 subset, specialized in cross-presentation of antigens to CD8$^+$ T-cells and stimulating Th1 immunity (Bachem et al., 2010; Jongbloed et al., 2010). The cDC2 subset was identified as murine CD11b$^+$/ human CD1c$^+$ cells (Haniffa et al., 2013), and is particularly capable to stimulate Th2 and Th17 immunity (Dutertre et al., 2014; Tussiwand and Gautier, 2015). Both pDC and cDC arise from a common DC progenitor in the bone marrow (Liu and Nussenzweig, 2010; Onai et al., 2007) and are dependent on the growth factor fms-like tyrosine kinase 3 ligand (Flt3L) for their development and differentiation (Karsunky et al., 2003; Schmid et al., 2010). Accordingly, all DC subsets express Flt3 receptor (CD135).

A number of reports described the presence of distinct cDC subpopulations in veterinary species, including pigs (Guzylack-Piriou et al., 2010; Maisonnasse et al., 2015; Summerfield et al., 2015; Auray et al., 2016, submitted), cattle (Howard et al., 1999; Renjifo et al., 1997) and sheep (Contreras et al., 2010; Pascale et al., 2008). Additionally, pDC have been identified in pig (Summerfield et al., 2003), in cattle (Reid et al., 2011) and in sheep (Pascale et al., 2008).

However, neither cDC nor pDC have been described in horses. Studies that have been performed to date, resorted to the use of monocyte-derived dendritic cells (MoDC) (Cavatorta et al., 2009; Mauel et al., 2006; Moyo et al. 2013). Although MoDC possess many functional

attributes consistent with DC (Sallusto and Lanzavecchia, 1994), they do not represent *bona fide* DC based on their ontogeny. Nevertheless, many *in vivo* studies report MoDC as a distinct subtype of inflammatory monocytic cell, sharing some features with cDC in terms of antigen presentation (Guilliams et al., 2014; Schlitzer et al., 2015). Consequently, for advances in immunological research in horses, it is essential to gain more information on equine *bona fide* DC.

The aim of the present study was to phenotypically characterize equine blood DC and functionally identify pDC in healthy horses. As a first step, appropriate cell surface markers which were either equine-specific or shown to cross-react with the equine system were used to identify distinct cell subsets in equine peripheral blood mononuclear cells (PBMC) by flow cytometry. Based on the comparative approach used by Summerfield et al. (2015), a first classification of equine DC subsets is proposed. The identity of purified pDC was further confirmed by a robust IFN-α production upon TLR9-stimulation as well as by a high expression of pDC-specific transcripts.

2. Methods

2.1.Isolation of peripheral blood mononuclear cells from healthy horses

Blood samples were collected from the jugular vein of eleven healthy horses (age = 4 – 21 years) using sterile glass bottles supplemented with 5000 I.U./ml heparin (Liquemin®, Drossapharm AG, Basel, Switzerland) or Sodium-Heparin containing vacutainers (Vacuette®; Greiner, St.Gallen, Switzerland). The study was approved by the Animal Experimental Committee of the Canton of Berne and Vaud, Switzerland (No. BE 51/13).

PBMC were isolated by density gradient centrifugation over Biocoll (ρ=1.077 g/ml, Biochrom GmbH, Berlin, Germany) as described (Hamza et al., 2007).

2.2. Production of recombinant bovine Flt3 Ligand

Bovine instead of equine Flt3L was used because the complete equine Flt3L gene sequence is still unknown. The partial equine Flt3L sequence (Genbank: XP_005596791.1) exhibits 73% amino acid sequence homology to bovine Flt3L. Bovine Flt3L (NCBI NM_181030.2) was produced as previously described (Guzylack-Piriou et al., 2010) and was originally employed for another study (Baumann et al., unpublished data). Briefly, after deletion of the stop codon, the Flt3L sequence was flanked by HindIII and XbaI restriction sites and was chemically synthesized in pUC57 plasmid (GenScript, Piscataway, NJ, USA). After HindIII and XbaI digestion, Flt3L was ligated in the pEAK8-His expression vector. The TOP10 Chemically Competent *E. coli* cells (Invitrogen, USA) were transformed with the plasmid pEAK8-His containing the bovine Flt3L. For final recombinant production of bovine Flt3L, HEK 293 cells were transfected with pEAK8-His-Flt3L using X-tremeGENE 9 following the manufacturer's instructions (Roche, Basel, Switzerland). After 5 days, supernatant was collected and expression of recombinant bovine Flt3L was assessed by western blot using an anti-his-HRP antibody (cat. no. 130-092-785, Miltenyi Biotec; Antibody Register: AB_1103231). The reaction was visualized with a WesternBright ECL Western blotting detection kit (Advansta Inc., Menlo Park, CA, USA) and a CCD-LAS3000 camera (Fuji Film) (Fig. S1).

2.3. Surface marker analysis by flow cytometry

PBMC from three horses were transferred to 5ml FACS tubes at 3 x 10^6 cells per tube. First, a blocking step was performed using Chrome Pure whole mouse IgG (Jackson Immunoresearch, West Grove, PA, USA). All incubations were performed for 20 min on ice, followed by washing with PBS and centrifugation at 500 x g for 5 min. Briefly, cells were incubated with recombinant bovine Flt3L, followed by labelling with a PE-conjugated anti-his antibody (clone GG11-8F3.5.1, cat-no 130-092-691; Miltenyi Biotec; Antibody Register: AB_1103227). Anti-human CADM-1 (cat no. CM004-3, MBL International Corporation;

Antibody Registry: AB_592783), shown to specifically bind to the CADM-1 molecule in many mammalian species (Contreras et al., 2010; Dutertre et al., 2014), was detected by a secondary goat biotinylated anti-chicken antibody (cat no 103-065-155, Jackson Immunoresearch; Antibody Registry: AB_2337383), followed by labelling using Brilliant Violet (BV)421 conjugated to Streptavidin (BD Biosciences, Franklin Lakes, NJ, USA). Other surface markers were stained using the following antibodies: anti-equine CD4 (clone CVS4; cat no MCA1078, Bio-Rad; Antibody Register AB_321274**)**, anti-equine CD13 (clone CVS19; cat no MCA1084GA, Bio-Rad; Antibody Registry**:** AB_321308), anti-equine MHCII (clone CVS20; cat no MCA1085, BioRad; Antibody Register AB_321618**)**, anti-equine CD14 (clone 105; https://courses2.cit.cornell.edu/wagnerlab/research/reagents.htm; Kabithe et al., 2010)), anti-bovine CD172a (clone HR-DH59B; cat no HR-BOV2049; Monoclonal Antibody center, Washington State University; Pullman WA, USA) showing cross-reactivity with equine cells (Mérant et al., 2009). These monoclonal antibodies were labelled with mouse IgG1 Alexa Fluor 488, 647 or 700 Zenon labelling kits (Thermo Scientific, Waltham MA, USA). Appropriate isotype and fluorescence-minus-one (FMO) controls were used. Finally, cells were resuspended in phosphate buffered saline (PBS) and analysed on a LSRII flow cytometer (BD Biosciences). Automated compensation for spectral overlap of fluorochromes was calculated by the BD FACSDIVA acquisition software (BD Biosciences) based on single-stained PBMC. Data were analysed using FlowJo software 6 (Tree Star Inc. Ashland OR, USA). Gates were set to exclude doublets and lymphocytes based on forward and side scatter characteristics (Fig. 1A). Within this cell population, a further gate was set on $Flt3^{+}$/ $CD14^{low}$ cells, based on isotype and fluorescence minus one (FMO) controls. This gating strategy was used for all flow cytometry experiments.

2.4. PBMC stimulation for IFN-α production

PBMC were isolated as described above and suspended in RPMI 1640 medium with HEPES and L-glutamine (Gibco, Life Technologies Ltd, Paisley UK) supplemented with 1% penicillin and streptomycin (Gibco), 1% MEM vitamins, 1% Na pyruvate, 1% Non-essential amino acids (all Biochrom GmbH) and 10% inactivated horse serum (Ziegler et al. 2016, in revision) at a density of 2 x 10^5 cells per 200 µl medium in a 96-well round-bottom cell culture plate (Sarstedt, Nümbrecht, Germany). Cells were cultured for 24h in the presence of 5 µg/ml of the synthetic TLR9 agonist Type C CpG-oligodeoxynucleotides (ODN) D-SL03 (InvivoGen, San Diego, CA, USA) or of equine herpesvirus-1 (EHV-1; MOI of 0.04 $TCID_{50}$/ cell, EHV1-V144/64, kindly supplied by Prof. Reto Zanoni from the Institute for Virology and Immunology, Vetsuisse Faculty, University of Berne, Switzerland). Thereafter, cell culture supernatants were collected and stored at -80°C until used.

2.5. Enrichment of pDC by depletion of PBMC from $CD5^+$ and $CD14^+$ cells

PBMC were depleted of $CD5^+$ and $CD14^+$ cells by magnetic separation (MACS technology, Miltenyi Biotec GmbH) according to standard protocols by the manufacturer, using a monoclonal anti-equine CD5 antibody (clone CVS5; cat no MCA1079GA, Bio-Rad; Antibody Register: AB_321382) and anti-equine CD14 (clone 105, Kabithe et al., 2010). Briefly, PBMC were first incubated with anti-CD5 and anti-CD14 simultaneously and, after a washing step, with secondary goat anti-mouse micro beads. Cells were then separated on a LD column (Miltenyi Biotec GmbH). After washing, the CD5/ CD14-depleted (purity > 75%) and CD5/ CD14-enriched (purity > 95%) fraction were stimulated with CpG-ODN or EHV-1 as described above for 24h and supernatants were harvested and stored at -80°C until used.

2.6. Sorting experiments

$Flt3^+$ cells from three horses were enriched from PBMC by MACS separation as described above, using the recombinant bovine Flt3L bound by an anti-his antibody conjugated to PE

and ultra-pure anti-PE microbeads (both Miltenyi Biotec GmbH). The magnetic separation was performed at 4°C. Following Flt3-enrichment, cells were stained for expression of CD14, CADM-1 and MHCII as mentioned above. Using a BD FACSAria sorter, $Flt3^{+}CD14^{low}$ cells were gated. $MHCII^{low}$/ $CADM\text{-}1^{-}$, $MHCII^{high}$/ $CADM\text{-}1^{low}$ and $MHCII^{high}$/ $CADM\text{-}1^{+}$ subpopulations were identified and sorted. Sorted cells were used for subsequent stimulation with the TLR9 agonist CpG-ODN D-SL03, as described above, using 20'000 cells/ 100 µl medium per well. The remaining cells were resuspended in 1ml Isol-RNA Lysis Reagent (5 Prime, Hilden, Germany) and kept at -80°C until used.

2.7. Equine IFN-α ELISA

For detection of IFN-α in cell culture supernatants, we used an equine IFN-α ELISA (kindly supplied by Dr. Bettina Wagner, Cornell University, Ithaca NY, USA). The assay was performed as described (Wagner et al., 2008). Briefly, anti-IFN-α (clone 29B; https://courses2.cit.cornell.edu/wagnerlab/research/reagents.htm) was used for coating of the plates at a concentration of 5 µg/ml. rIFN-a/IgG4 supernatant (85 ng/ml) was serially diluted two-fold, ranging from 42.5 ng/ml – 0.66 ng/ml, to obtain a standard curve. Cell culture supernatants of stimulated cells were tested in duplicates at a 1:2 dilution. For detection, biotinylated anti-IFN-α (clone 240; https://courses2.cit.cornell.edu/wagnerlab/research/reagents.htm) was used. This step was followed by incubation with streptavidin-peroxidase and TMB substrate (Sigma-Aldrich GmbH, Buchs, Switzerland). Optical density was measured at 450 nm on an ELISA Reader (BioTek Instruments Inc., Winooski VT, USA) and IFN-α concentration in the samples was calculated according to the standard curve calculated by the Gen5 software (BioTek Instruments Inc.).

2.8. Quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR)

For RNA extraction from sorted PBMC fractions, 200 µl of chloroform:IAA (49:1; Sigma-Aldrich GmbH) were added to 1 ml of Isol-RNA lysate and tubes were vigorously shaken by hand. After 5 min incubation at room temperature, samples were centrifuged at 12,000 x g for 15 min. The aqueous upper phase was subsequently transferred to a fresh tube and 500 µl of 100% isopropanol were added. Precipitated RNA was then loaded onto a spin column from RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) and purification was performed according to manufacturer's instructions. RNA was eluted in 40 µl of RNAse-free H_2O. Each sample was quantified spectrophotometrically (NanoDrop 1000, Thermo Scientific) and stored at -80 °C until used. A total of 9 µl of RNA was employed to synthesize cDNA using GoScript™ Reverse Transcription system (Promega, Madison WI, USA) following manufacturer's instructions. Expression of *PLAC8*, *RUNX2*, *TCF4* and *BACT* were quantified by qPCR using the GoTaq® qPCR Master Mix (Promega) with the primers listed in the table S1. The reaction was performed in a total volume of 25 µl in the 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) under the following fast-cycle amplification conditions: 2 min at 95°C, followed by 40 cycles of denaturation at 95°C for 3 seconds followed by annealing/ extension at 58°C for 30 seconds. The specificity of each primer pair was confirmed in melting-curve analysis and primer efficiency reached > 90% by using serial cDNA dilution as template. Relative expression was normalized to the housekeeping gene *BACT* expression and ΔCt was calculated as: $2^{-Ct\ gene} / 2^{-Ct\ housekeeping\ gene}$.

2.9 Statistical Analysis

Statistical analysis was carried out using the software program NCSS8. As the data were not normally distributed, a non-parametric Wilcoxon (signed rank) test was used to compare levels of secreted IFN-α between stimulation conditions. P-values ≤ 0.05 were considered significant.

3. Results and Discussion

3.1. Surface marker expression of equine dendritic cell subsets

Since the Flt3 expression on DC is well conserved in mammals (Karsunky et al., 2003; Schmid et al., 2010; Summerfield et al., 2015), Flt3L constitutes a valuable tool to identify DC in a mixed cell population. Orthologous bovine Flt3L was shown to bind to a small heterogeneous population of equine PBMC (0.42 – 0.85%) which did not express CD14 at all, or at very low levels only (Fig 1B).

Within the $Flt3^{+}$/ $CD14^{low}$ gate, three subpopulations (P4, P5 and P6) could be further identified based on the differential expression of MHCII and CADM-1 (Fig. 1C). P4 was found to express the lowest levels of CD172a, while P5 expressed high levels, comparable to monocytes (Fig. 1D). None of the subsets was found to express high levels of CD4 (Fig. 1D). Interestingly, CD13 was co-expressed with CADM-1 on a population within $Flt3^{+}CD14^{low}$ cells (Fig. 1D). In alignment with other species and considering that CADM-1, CD13, Flt3 and CD172a represent cell surface receptors with a species-conserved expression (Summerfield et al., 2015), we propose that equine cDC1 can be identified as $Flt3^{+}CD4^{-}$ $CD13^{+}CD14^{low}CD172a^{-}CADM\text{-}1^{+}MHCII^{high}$ cells, while equine cDC2 would be $Flt3^{+}CD4^{-}$ $CD13^{-}CD14^{low}CD172a^{+}CADM\text{-}1^{low}MHCII^{high}$. This will require future confirmation for example by employing transcriptional profiling of the sorted populations. As the present study was focusing on pDC we continued our experimentation focusing on the $Flt3^{+}CD4^{-}$ $CD13^{-}$ $CD14^{-}CD172a^{-}CADM\text{-}1^{-}MHCII^{low}$ subset as a possible candidate for pDC.

3.2. Functional identification of equine pDC

As a first experiment, PBMC from eleven healthy horses were stimulated with the synthetic TLR9 agonist type C CpG-ODN or with EHV-1 and the amount of IFN-α in the supernatants was quantified by ELISA. A significant IFN-α induction could be observed in cells stimulated with both EHV-1 and CpG-ODN compared to unstimulated cells (Fig.2A). Intriguingly, while

an induction of IFN-α by EHV-1 was shown in PBMC from all horses, five out of the eleven tested horses were non-responsive to stimulation by CpG-ODN (Fig. 2A). The strongest induction of IFN-α in response to CpG-ODN was detectable with PBMC of horses, which also released the highest quantities of IFN-α in response to EHV-1. We speculate that there was a higher number of pDC present in the PBMC of these horses. On the other hand, EHV-1 contains a number of pathogen-associated molecular patterns. It may have been able to induce IFN-α production through a broader stimulation of various pathways, possibly also in other cells than pDC, thus inducing IFN-α secretion also in PBMC of horses which were unresponsive to CpG-ODN stimulation. In order to enrich the pDC population, PBMC were depleted of $CD5^+$ T-cells and $CD14^+$ monocytes. Whereas CD5-/CD14-depleted PBMC released high levels of IFN-α, no response was observed in CD5-/CD14-enriched fractions after CpG or virus stimulation (Fig. S2). These data indicate that the pDC do not express CD5 and CD14.

To further confirm pDC identity and to obtain sufficient cell numbers for FACS sorting, $Flt3^+$ cells were enriched by MACS sorting, resulting in 7% $Flt3^+$ cells (range 3 – 10.1%), which represented an 11-fold enrichment (data not shown). Similar to what we observed with PBMC, three cell subsets could be clearly distinguished in the $CD14^{low}$/ $Flt3^+$ fraction with regard to MHCII and CADM-1 expression (Fig. 2B). They were subsequently sorted by FACS. Stimulation of PBMC, Flt3 MACS-enriched and Flt3 MACS-depleted fractions with CpG-ODN revealed distinct differences in IFN-α induction, confirming the presence of pDC in the $Flt3^+$ cells (Fig. 2C). Slight increase in IFN-α secretion was observed in the Flt3 MACS-enriched compared to PBMC (median; range = 14.1; 9.1 – 22.2 ng/ml and 9.7; 0.23 – 11.9 ng/ml, respectively), whereas no IFN-α production could be detected in the Flt3-depleted fraction. As hypothesized, IFN-α was solely produced by the P4 fraction of putative pDC (median 20.2; range 15.9 – 50.3 ng/ml), with no IFN-α production detected in the other two subsets, indicating that the P4 subset would represent pDC.

Recent breakthroughs in transcriptomic profiling of DC subsets have revealed cell-type specific transcripts some of which were conserved between species (Miller et al., 2012; Shay et al. 2013). Thus, new possibilities have opened up for characterization of DC subsets in veterinary species (Summerfield et al., 2015; Vu Manh et al., 2015). Moreover, expression analysis of genes known to be specifically expressed in pDCs in other species including *PLAC8* (=*C-15*) (Rissoan et al., 2002), *RUNX2* (Sawai et al., 2013) and *TCF4* (= *E2-2*) (Cisse et al., 2008) revealed a high expression in the putative equine pDC fraction (Fig. 2D). While *RUNX2* levels were found to be low in the P5 cell fraction, no expression of the other transcripts was detected, indicating that *PLAC8* and *TCF4* represent pDC-specific transcripts also in horses. We found a similar restricted expression of these genes in porcine pDC (Auray and Summerfield, unpublished results). While the function of *PLAC8* on pDC is unknown, *RUNX2* and in particular *TCF4* represent essential transcription factors for their development (Sawai et al., 2013; Cisse et al., 2008). Considering the transcriptions factors controlling immune cell development are well conserved, this data confirms that the $Flt3^{+}CD4^{-}$ $CD13^{-}$ $CD14^{-}CD172a^{-}CADM\text{-}1^{-}MHCII^{low}$ P4 subset represents or contains equine pDC.

In summary, we have identified equine pDC and propose a basis for further characterization of equine blood cDC. Alignment of the phenotype of equine pDC with other species highlighted some intriguing differences, such as a very low or lacking expression of CD4, contrasting with several other mammals (Summerfield et al., 2015). Considering the importance of DC in many immunological processes, future work should follow to identify DC-subset specific transcripts, which will be the basis to establish reagents for equine DC characterization.

4. Conflicts of interest

The authors declare no conflicts of interest

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Figure Legends

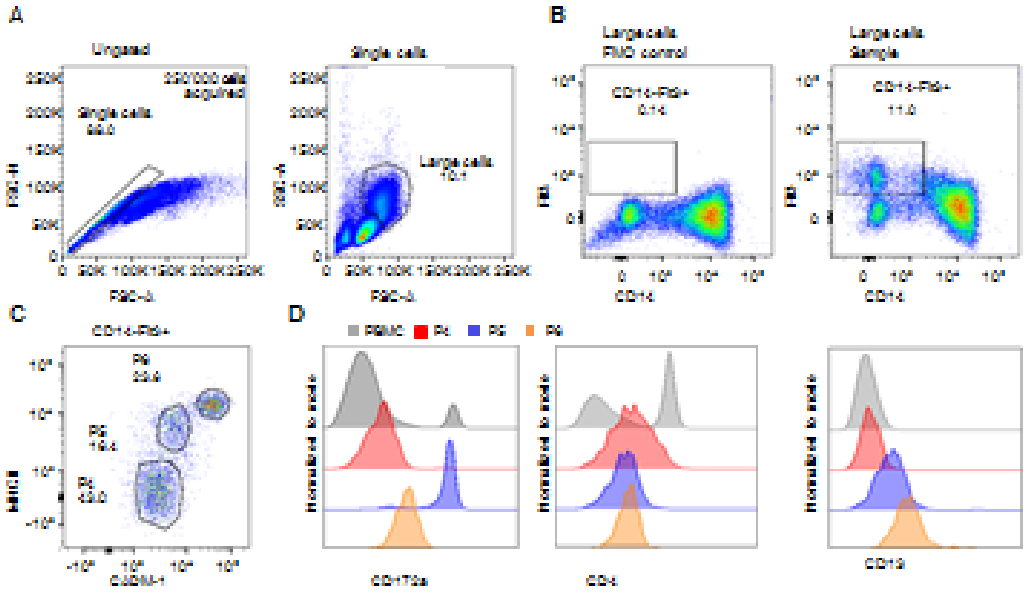
Figure 1.

Phenotype of Flt3^{+} cells in equine PBMC. (A) Gating strategy to remove doublets and include FSC^{high}/ SSC^{high} large cells. A total number of 250'000 cells were acquired. (B) Within the single, large cell gate, based on the fluorescence minus one (FMO) control, a gate on Flt3^{+}CD14^{-} cells was set. (C) Identification of three putative DC subpopulations based on MHCII and CADM-1 expression within Flt3^{+}CD14^{-} gated cells. (D) Histograms showing the expression levels of CD172a, CD4 and CD13 in P4 (red; pDC), P5 (blue; cDC2) and P6 (orange; cDC1) compared to PBMC (grey). Data are shown for one representative animal out of three.

Figure 2.

Characterization of equine pDC. (A) IFN-α secretion by equine PBMC upon stimulation with CpG-ODN (5 µg/ml) or EHV-1 (MOI of 0.04 $TCID_{50}$/cell). Samples were tested in duplicates and results are displayed as mean optical density (OD) measured at 450 nm. Each symbol indicates a separate horse with red lines indicating the median. A non-parametric paired sample Wilcoxon signed rank test was performed to compare the stimulation conditions. P-values ≤ 0.05 were considered statistically significant as indicated by asterisks. (B) Gating strategy of putative DC subsets within Flt3-enriched cells which were employed for FACS. A gate was set on the Flt3^{+}/ CD14^{low} cells. The three distinct cell populations in the CADM-1/ MHCII plot were then gated and sorted. (C) IFN-α was detected in cell culture supernatants of PBMC (white background), Flt3-enriched and Flt3-depleted fractions, respectively (light green background) as well as FACS-sorted DC subsets (red background) stimulated with 5 µg/ml CpG-ODN for 24h. Each symbol represents an individual horse with red lines indicating the median. Samples were tested in duplicates and results are displayed as ng/ml. (D) Relative expression of *PLAC8*, *RUNX2* and *TCF4* transcripts in the sorted populations P4,

P5 and P6. The expression was normalized to the housekeeping gene *BACT*. Bars show the mean +/- SD of an independent experiment performed in duplicate. One representative sorting out of three is shown.


A
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Single cells
B
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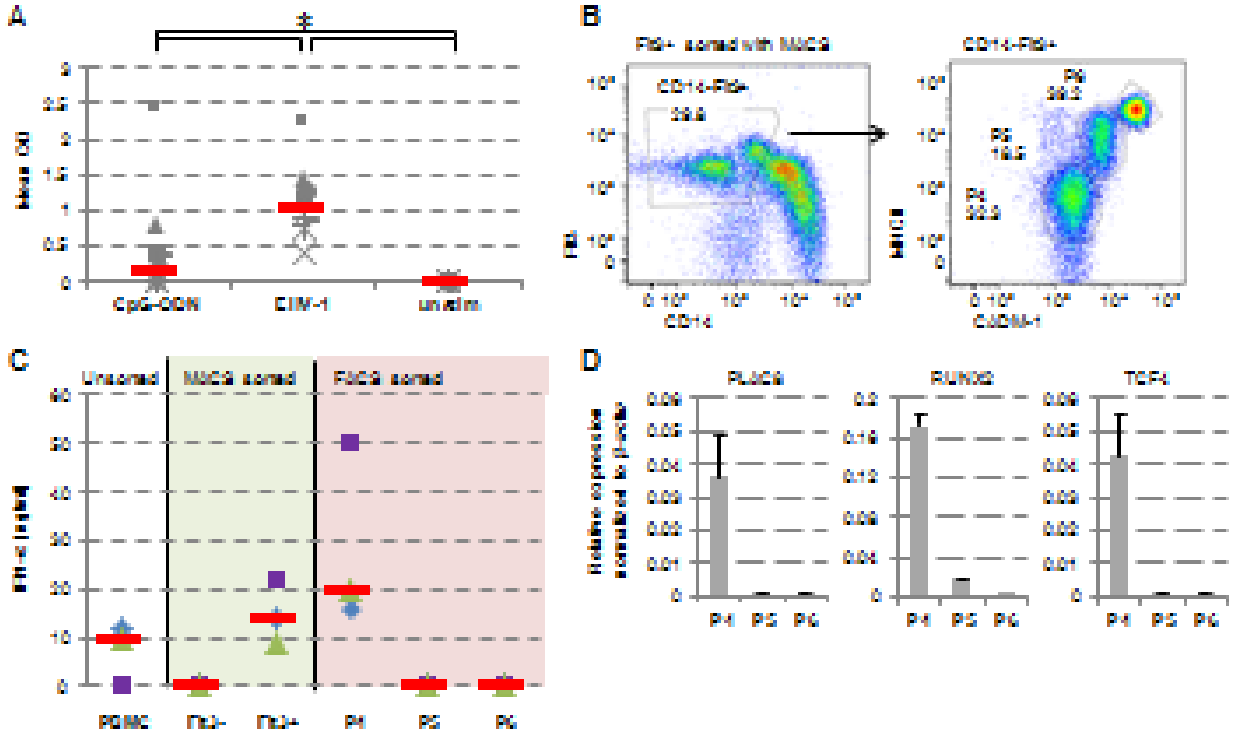
FMO control
Large cells
Sample
C
D
PBMC
P1
P2
P3
CD1T2a
CD4
CD19

457
458


A
CpG-ODN
CIM-1
unstim
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CD14+-Fib+
CD14
CD90.1
C
Unsorted
MACS sorted
FACS sorted
PBMC
Fib-
Fib+
P1
P5
P6
D
PLSCR
RUNX2
TCF4
P1
P5
P6

459

460

Table S1. Primers used for qRT-PCR.

Transcription Factor Gene	Primer	Sequence
PLAC8	Forward	5′ GCCAGTGGTCATTGTGACTC
	Reverse	5′ GATCCAGGGATGCCATATCG
RUNX2	Forward	5′ GGCAAGAGTTTCACCTTGAC
	Reverse	5′ GAATGCGCCCTAAATCACTG
TCF4	Forward	5′ CCACCTCAAGAGTGACAAAC
	Reverse	5′ TTTCAGACACGCAGCTTTCG
BACT	Forward	5′ CACCACACCTTCTACAAC
	Reverse	5′ ATCTGGGTCATCTTCTCG

Table S2. Proposed classification of equine dendritic cell subsets in the blood based on surface marker expression.

DC subset	Flt3	CD4	CD14	CD172a	CD13	CADM-1	MHCII
cDC1	+	-	(-)	-	+	+	++
cDC2	+	-	(-)	+	-	-	++
pDC	+	(-)	-	-	-	-	+

Figure S1. Recombinant bovine Flt3L expressed in HEK293 cells was visualized by Western blotting. A total of 20 µl of 10-fold diluted supernatant was loaded on the gel. The Flt3L was detected with an mouse anti-His-HRP antibody (Miltenyi Biotec, Antibody Register: Antibody Register: AB_1103231) and visualized with a WesternBright ECL Western blotting detection kit (Advansta Inc., Menlo Park, CA, USA)